AD_____

Award Number: DAMD17-03-1-0338

TITLE: Summer Student Breast Cancer Research Training Program

PRINCIPAL INVESTIGATOR: Gary P. Zaloga, M.D.

CONTRACTING ORGANIZATION: Clarian Health Partners, Incorporated
Indianapolis, IN 46206-1367

REPORT DATE: May 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2005	Annual Summary(15 Apr 2004 - 14 Apr 2005)

4. TITLE AND SUBTITLE
Summer Student Breast Cancer Research Training Program

5. FUNDING NUMBERS
DAMD17-03-1-0338

6. AUTHOR(S)
Gary P. Zaloga, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Clarian Health Partners, Incorporated
Indianapolis, IN 46206-1367

E-Mail: gzaloga@clarian.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The purpose of the breast cancer research training program is to recruit and train potential future researchers in breast cancer research. From a total of 90 applications for summer research, we selected a total of five students. During the 12-week program, trainees attended seven lectures, participated in weekly research and lab meetings, and developed and carried out research projects under the supervision of a mentor. Two projects addressed the effects of omega-3 lipids upon breast cancer cells. Omega-3 lipids were found to decrease breast cancer-induced muscle cell proteolysis and to induce apoptosis in cancer cells. Methyl and ethyl forms of omega-3 lipids failed to induce apoptosis. Ganoderma lucidum, a Chinese mushroom, was found to inhibit breast cancer cell growth and decrease EGF receptor phosphorylation. C-reactive protein (CRP) and a CRP peptide were found to upregulate urokinase plasminogen activator in cancer cells. The final study evaluated the effect of inhibitors of the mitogen activated protein kinases. A specific inhibitor of ERK increased apoptosis in highly invasive breast cancer cells. The results of the summer projects contributed significantly to ongoing breast cancer research at Methodist Research Institute and have added new information to our understanding of breast cancer development and progression.

14. SUBJECT TERMS
Undergraduate research training, breast cancer, fatty acids and prevention, nutrition and prevention, alternative prevention

15. NUMBER OF PAGES
125

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

The year 2003 to 2004 was the second year of the Methodist Research Institute's Breast Cancer Research Training Program funded by the US Army Medical Research and Materiel Command. The purpose of the Breast Cancer Research Training Program is to recruit, train, and provide the opportunity for well-qualified college science and premedical students to work on a biomedical research project in breast cancer with a medical researcher. Our goal is to instill in the students a passion for and commitment to breast cancer research as a career. This training was accomplished during the months of May through August 2004.

This report describes the training and research accomplishments of the 2004 program, including reportable outcomes such as manuscripts, abstracts, and presentations. The first section of the body of the report will describe the students and preceptors, and their research projects. The next section will list training accomplishments, and the final section will define the reportable outcomes of the program. The appendix includes manuscripts, an abstract presented orally at the 2005 Experimental Biology meeting, abstracts of the projects, printouts of the presentations from Presentation Day, and the lecture schedule.

Body

Planning for the Breast Cancer Research Training Program began in November with development of application materials and student recruitment. The application due date was February 16 and students were offered positions in late March to early April. Students began the program on May 24th and the program ended August 13th. Presentation Day was moved up to August 6 because first-year medical students needed to leave the following week for orientation to their medical school programs.

Eleven applicants to the 2004 program expressed a specific interest in breast cancer research. Thirteen other applicants expressed an interest in cancer research and were also considered for the Breast Cancer Research Training Program. Three of the applicants to the program were from underrepresented minorities. We interviewed seven candidates for two positions in the program (three students from the 2003 program returned for a second year in the 2004 program).

The following list presents the students who were ultimately chosen, the college or university attended, their class status at the time of their participation, and their BCRT program preceptor(s):

Student	Class and Institution	Preceptor
Brian Bock	Senior, Valparaiso University	Daniel Sliva
Jennifer Griffith	First Year, Washington University School of Medicine	Thomas Kovala
Laura Sech	First Year, Indiana University School of Medicine	Rafat A. Siddiqui/Gary P. Zaloga
Chelimo Yego (minority)	Graduate, Eastern University	Carlos A. Labarrere
Heidi Yount	First Year, Wright State University School of Medicine	Rafat A. Siddiqui/Gary P. Zaloga

Three of these students had been accepted into medical school, one of them in the MD/PhD Program at Washington University in St. Louis.

Training Accomplishments According to the Statement of Work

Task 1: Recruitment of undergraduate students interested in breast cancer research training

- October 2003: Program application updated and information materials prepared and uploaded onto the website
- October through November 2003: Information packages with application materials to students, colleges/universities, and community leaders mailed. Application packages and brochures were mailed out in response to requests for applications.
- December 2003: Follow-up phone calls were made and brochures were sent out to promote the Breast Cancer Research Training Program at Methodist Research Institute.

Task 2. Selection of students to participate in the Breast Cancer Research Training Program

- February 17, 2004: All applications received by February 17, 2004 or with a postmark of February 17, 2004 were considered for the program.
- March 5, 2004 to March 24, 2004: The Selection and Interviewing Committee chose prospective candidates and interviewed them.
- March 24, 2004: Interviews with students were completed and committee members chose two students to participate in the Breast Cancer Research Training Program and matched them with BCRTP preceptors.
- March 24 to April 1, 2004: Selected students were notified of their acceptance to the program.

Task 3: Breast Cancer Research Training Program

- May 24, 2004: Students began the Methodist Research Institute Breast Cancer Research Training Program.
- Students attended seven lectures between June 4, 2004 and July 30, 2004. See appendix for lecture topics and schedule.
- From May 26, 2004 to August 4, 2004, students conducted breast cancer research under the guidance of their preceptors. This training included weekly meetings of all students and preceptors in the program.
- Students attended a lecture on writing research papers; preceptors guided their students in writing their research papers.
- Preceptors guided students in their preparation of oral presentations; students presented their projects informally to other students at weekly meetings.
- August 5, 2004: All students participated in rehearsal for Presentation Day.
- August 6, 2004: All students delivered oral presentations of their projects to an audience of researchers at Methodist Hospital.
- August 6, 2004: All students turned in their research papers.

Because three of the five students in the Breast Cancer Research Training Program were entering medical school in August, Presentation Day had to be moved up by one week to accommodate

their orientation schedules. However, students were encouraged to continue work on their projects for the duration of the program until August 13, 2004.

Task 4. Evaluation of the program

- August 5, 2004: Students turned in evaluation forms for the program.
- August 19, 2004: Program Coordinator wrote a final evaluation of the program.
- September 2004: Program Director and Coordinator met to review the evaluation.
- *Program Director and Coordinator developed plans for improvement of the program based on the evaluation.*

Key Research Accomplishments

Research Accomplishments
The following paragraphs describe the projects undertaken in the 2004 Breast Cancer Research Training Program and the results of those projects.

Project 1. Inhibition of breast cancer growth with Ganoderma lucidum.
Brian Bock and Dr. Daniel Sliva, Cancer Research Laboratory at the Methodist Research Institute, investigated the activity of epidermal growth factor receptor (EGFR) after treatment of MDA-MB-231 and MCF-7 breast cancer cells with *Ganoderma lucidum (GL),* an ancient Chinese medicinal mushroom. Both types of cells are known to express some of the EGFR family of tyrosine kinase receptors. It was anticipated that phosphorylation levels of some EGFR family members would be decreased by treatment with *GL*. *GL* treatment was then applied to a nude mice animal model (no US Army MRMC funds were used to purchase animals or animal products for this project). Nude mice were injected with MDA-MB-231 breast cancer cells and randomized into three groups: control (placebo); 100 mg/kg *GL*; and 500 mg/kg *GL*. Results showed that 100 mg/kg was effective in inhibiting tumor growth compared to control. Results were inconclusive at 500 mg/kg because of the death of some of the mice. However, it can be concluded that *GL* can be a potential inhibitor of tumor growth in highly invasive breast cancer cells.

Project 2. Effect of kinase inhibitors on ERK activity in breast cancer cells.
Jennifer Griffith worked with Dr. A. Thomas Kovala to test the ability of various compounds to directly inhibit ERK activity in the mitogen-activated protein kinase (MAPK) pathway and to sensitize breast cancer cells to apoptosis. ERK has been found to be activated in two highly aggressive breast cancer cell lines: MDA-MD-231 and MDA-MB-468. In contrast, in relatively normal (MCF-10) and less invasive (MCF-7) cell lines, ERK activity is regulated normally. Three compounds were tested for their ability to change the regulation of ERK in the highly invasive breast cancer cell lines: AG1296; SU1498; and AG1007. AG1296 was found to inhibit ERK phosphatase activity, while ERK kinase activity was not affected. SU1498 was found to increase apoptosis in both highly aggressive breast cancer cell lines, though it is not uniformly effective. AG1007 did not induce an increase in apoptosis in any of the cell lines. Thus, SU1498 remains the kinase inhibitor with the greatest potential in cancer treatment.

Project 3. Omega-3 fatty acids, apoptosis, and membrane organization.
Laura Sech worked with Drs. Gary Zaloga and Rafat Siddiqui investigating the role of omega-3 fatty acids in inducing apoptosis in MDA-MB-231 breast cancer cells. In last year's project, Laura found that the most effective omega-3 fatty acid supplements showed high amounts of methylated forms of docosahexaenoic acid (m-DHA) and eicosapentaenoic acid (m-EPA). The present study tested the hypothesis that m-DHA and m-EPA may be more effective in inducing apoptosis in breast cancer cells than the nonmethylated forms. Results showed that, contrary to the hypothesis, omega-3 fatty acids in their free form seem to be more cytotoxic to breast cancer cells than either the methyl or ethyl forms. In a second study, the role of omega-3 fatty acids in phase separation from lipid rafts was examined in vivo. It has been shown using biophysical measurement in vitro that omega-3 fatty acids influence the organization of the plasma membrane by phase separating into lipid raft molecules. In the present study, it was hypothesized that various fatty acids would be localized into nonraft domains—as opposed to raft domains. This was determined using the biochemical detergent extraction method in breast cancer cells. In accordance with the hypothesis, EPA, retinol, and DHA were localized predominantly in the nonraft regions.

Project 4. Effects of C-reactive protein (CRP) and CRP peptide (RS83277) on activation of NF-κB and upregulation of uPA and uPAR.
C-reactive protein (CRP) is an acute-phase protein released in the liver in response to inflammation. Chelimo Yego and Dr. Carlos Labarrere hypothesized that the CRP peptide RS83277 increases MDA-MD-231 breast cancer cell growth by activating the nuclear factor-kappa B (NF-κB) and by subsequently upregulating urokinase plasminogen activator (uPA) and its receptor uPAR. The investigators concluded that CRP and CRP peptide RS83277 may promote the growth of breast cancer cells as secretion of uPA is increased. Activation of NF-κB will consequently promote the upregulation of uPA and uPAR, resulting in increased migration, adhesion, and invasion of cells.

Project 5. Role of the calpain proteolytic pathway in breast cancer-induced cachexia.
In a study continuing from the 2003 Summer Student Research Program, Heidi Yount and Drs. Gary Zaloga and Rafat Siddiqui examined whether two omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), inhibit the epidermal growth factor (EFG) receptor, and hence, activation of the calpain pathway. The calpain pathway is relevant to cancer research because it is the initial step for muscle catabolism, such as in cancer-induced cachexia. Skeletal muscle cells were treated with growth media alone and with growth media from MDA-MD-231 breast cancer cells. Results showed that DHA decreased proteolysis of skeletal muscle cells treated with media alone and media from MDA-MB-231 cells containing proteolysis-inducing factor. Gelatin zymography studies were not complete at the time, but the results were expected to show a decrease in the amount of calpain protease activity in the cells treated with DHA, supporting the conclusion that DHA inhibits proteolysis by modulating the calpain proteases. Results for the effect on activation of the EGF receptor were inconclusive.

Reportable Outcomes (Students' names are in bold; preceptors' names are in italics)

Manuscripts

Wu M, Harvey KA, Welch ZR, **Sech L,** Jackson K, Stillwell W, *Zaloga GP, Siddiqui RA.* Omega-3 fatty acids attenuate breast cancer growth through activation of a sphingomyelinase-mediated pathway. *Int J Cancer.* (in press).

Boguslawski G, McGlyn PW, **Griffith J,** Harvey KA, *Kovala AT.* SU1498, an inhibitor of vascular endothelial growth factor receptor 2 and ERK1/2, prevents dephosphorylation of phospho-ERK and promotes apoptosis. (under revision)

Abstracts

Student abstracts published in the 2004 Summer Student Research Program Presentation Day Program (see appendix).

Presentations

Siddiqui RA, Wu M, Harvey KA, Welch ZR, **Sech L,** Jackson K, Stillwell W, *Zaloga GP.* Omega-3 fatty acids attenuate breast cancer growth through activation of a sphingomyelinase-mediated pathway. *FASEB J.* 2004;19:A1693 (oral presentation at Experimental Biology Meeting). *Student's name left out in journal (see appendix)

Siddiqui RA, Wu M, Ruzmetov N, Harvey KA, Welch ZR, **Sech L,** Jackson K, *Zaloga GP,* Stillwell W. Neutral sphingomyelinase mediates inhibitory effects of omega-3 polyunsaturation on breast cancer development. To be presented at the American Institute for Cancer Research International Research Conference on Food, Nutrition, and Cancer, July 14-15, 2005, Washington, DC

Student presentations presented at the 2004 Summer Student Research Program Presentation Day (see appendix).

Conclusions

We recruited and selected 5 individuals for the 12-week Breast Cancer Research Training Program at the Methodist Research Institute (MRI). Trainees attended lectures dealing with research design, statistics, ethics, and research reporting. Trainees developed and worked on individual research projects under the supervision of a mentor. Each trainee significantly contributed to larger breast cancer research projects. Three of five projects dealt with dietary modulation of breast cancer cell growth and progression and showed that both Ganoderma lucidum (a Chinese mushroom) and omega-3 polyunsaturated fatty acids have anticancer effects. Another project evaluated the effects of C-reactive protein (a proinflammatory peptide) upon signaling pathways involved in cancer cell growth. CRP was found to promote signaling associated with tumor progression. A final study evaluated the effect of ERK inhibition upon cancer cell apoptosis. A specific ERK inhibitor promoted apoptosis. The knowledge obtained from these studies contributed significantly to ongoing studies at MRI and contributes to our knowledge of dietary effects upon breast cancer progression. One study has been accepted for publication and an additional submitted manuscript is being revised. A third manuscript dealing with muscle proteolysis is being prepared for publication. Three of our students have entered medical school this year. To date, they continue to maintain an interest in breast cancer.

APPENDICES

Student Abstracts from the 2004 Presentation Day Program

Student Presentations Delivered at the 2004 Presentation Day

Principal Investigator/Student Manuscripts & Presentation

2004 Summer Student Research Program Lecture Schedule

Methodist Research Institute

Summer Student Research Program's

PRESENTATION DAY 2004

ABSTRACTS

2004 PARTICIPANT ABSTRACTS

Brian Bock
Valparaiso University

Preceptor: Daniel Sliva, PhD
Cancer Research Laboratory,
Methodist Research Institute

INHIBITION OF BREAST CANCER GROWTH WITH *GANODERMA LUCIDUM*

Ganoderma lucidum (Reishi) is an ancient oriental medical mushroom that has been used for the treatment and prevention of many diseases, including cancer. The highly invasive (MDA-MB-231) breast cancer cells and the less-invasive (MCF-7) breast cancer cells are known to express some of the epidermal growth factor receptor (EGFR) family of tyrosine kinase receptors (EGFR/ErbB1/HER1, ErbB2/HER2, ErbB3/HER3). The biological effects of those receptors are exerted through the interaction with a specific ligand, which results in the phosphorylation of the EGFR family. The signaling is further mediated through phosphatidylinositol 3-kinase (PI3K) and mitogen-activated-protein kinase (MAPK) pathways. Both PI3K and MAPK activate the expression of transcription factors NF-κB and AP-1.

We have shown that treatment of MDA-MB-231 cells with *Ganoderma lucidum* inhibits the activity of NF-κB through the PI3K pathway. Inhibition of NF-κB leads to the inhibition of cell migration and proliferation, in which the EGFR family is involved. We are interested in looking at EGFR family activity after *Ganoderma lucidum* treatment.

Therefore, we treated MDA-MB-231 and MCF-7 cells with *Ganoderma lucidum* for different times (15, 30, 60, 120, and 240 minutes). Total proteins were extracted and the expression of EGFRs and phophorylated EGFRs was determined by Western blot analysis. It is anticipated that the level of phosphorylation of some of the EGFR family members will be decreased by treatment with *Ganoderma lucidum.*

The second part of the project was to apply the *Ganoderma lucidum* treatment to an *in vivo* trial. MDA-MB-231 cells were injected subcutaneously in nude mice. After tumor formation, the mice were randomized into three groups to receive either a placebo, 100 mg/kg of *Ganoderma lucidum*, or 500 mg/kg of *Ganoderma lucidum*. *Ganoderma lucidum* was administered by gavage every other day for a five week period. The volume of the tumors was measured three times a week and compared among the three groups. Compilation of volumes as a function of time showed inhibition of tumor growth in the 100 mg/kg group compared to the control ($p<0.023$). However, the 500 mg/kg group gave inconclusive results due to the death of some of the mice ($p<0.297$). Therefore, *Ganoderma lucidum* can be regarded as a potential inhibitor of the growth of highly invasive human breast cancers.

Brian's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the Department of the Army Medical Research and Materiel Command.

Brian is a senior Biology & Chemistry major at Valparaiso University. He is in the process of applying to medical school.

2004 PARTICIPANT ABSTRACTS

Jennifer Griffith
Indiana University—Bloomington

Preceptor: A. Thomas Kovala, PhD
Experimental Cell Biology Laboratory, Methodist Research Institute

EFFECT OF KINASE INHIBITORS ON ERK ACTIVITY AND APOPTOSIS IN BREAST CANCER CELLS

Signaling pathways are responsible for many cellular processes, including growth, proliferation, and apoptosis. Tumorigenic phenotypes may develop when the regulation of these pathways is disrupted. One such pathway often implicated in human cancers is the mitogen-activated protein kinase (MAPK) pathway, which includes the signaling molecules Ras, Raf, MEK, and ERK. In two highly aggressive breast cancer cell lines (MDA-MB-231 and MDA-MB-468), ERK is constitutively activated, while the relatively normal (MCF-10A) or poorly tumorigenic (MCF-7) cell lines have normal regulation of ERK activation. A compound with the ability to change the regulation of ERK would therefore have potential in the treatment of cancer. The kinase inhibitor SU1498 has been found to cause an accumulation of phosphorylated ERK by blocking the dephosphorylation of ERK and by directly inhibiting ERK activity. SU1498 also sensitizes breast cancer cells to apoptosis. Based on the observation that AG1296, another kinase inhibitor, also causes accumulation of hyperphosphorylated ERK, we hypothesized that AG1296 would also directly inhibit ERK activity and sensitize breast cancer cells to apoptosis induced by serum starvation. An assay for ERK activity, which measured the amount of phosphorylated Elk-1 product, showed that AG1296 did not directly inhibit ERK. Several kinase inhibitors structurally similar to either SU1498 or AG1296 were also screened for the ability to inhibit ERK. Of those screened, only AG1007 showed significant inhibition of ERK. The potential of the inhibitors SU1498 and AG1007 to sensitize cells to apoptosis was also tested. As was previously known, SU1498 increased apoptosis induced by serum starvation in the highly invasive MDA-MB-231 cells, while causing no change in the poorly invasive MCF-7 cells. SU1498 was also found to have no effect on the relatively normal MCF-10A cells, and to slightly increase apoptosis in MDA-MB-468 cells. In contrast, none of the four cell lines showed a significant increase in apoptosis when treated with AG1007. From these results, we conclude that the accumulation of hyperphosphorylated ERK that occurs upon treatment with AG1296 is probably due to inhibition of ERK phosphatase activity, while ERK kinase activity is not affected. Therefore this compound is not likely to be effective in increasing apoptosis in breast cancer cells. SU1498 was found to increase apoptosis in both highly aggressive breast cancer cell lines, though it is not uniformly effective. This effect is unique to the compound SU1498, since treatment with another ERK inhibitor, AG1007, did not induce an increase in apoptosis in any of the cell lines. Thus, SU1498 remains the kinase inhibitor with the greatest potential in cancer treatment.

Jennifer's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the Department of the Army Medical Research and Materiel Command.

Jennifer graduated from Indiana University this May...at least, we think she did, since a diploma has yet to be seen. In any case, beginning next week you can find her in St. Louis, where she will be pursuing an MD/PhD dual degree at Washington University School of

Medicine for the next eight (or nine, or twelve) years. She promises to treat all her visitors to a slice of St. Louis' finest gooey butter cake and a tour of Forest Park. As a three-time veteran of the SSRP, Jennifer would like to thank the researchers and staff at MRI for a wonderful experience and to assure them that this summer really will be her last.

2004 PARTICIPANT ABSTRACTS

Laura Sech
Indiana University School of Medicine

Preceptors: Rafat Siddiqui, PhD and Gary Zaloga, MD
Cellular Biochemistry Laboratory
Methodist Research Institute

OMEGA-3 FATTY ACIDS, APOPTOSIS, AND MEMBRANE ORGANIZATION

INTRODUCTION: It is becoming increasingly clear that dietary intake of omega-3 fatty acids may have profound health benefits for various disease states, including cancer. We investigated two distinct hypotheses regarding the role of omega-3 fatty acids in their ability to induce apoptosis of MDA-231 breast cancer cells. (i) Based upon a previous investigation, it was concluded that certain compositions of ω-3 fatty acid supplements were more effective in killing breast cancer cells. A molecular species analysis revealed that the most effective supplements showed high amounts of the methylated forms of the omega-3 fatty acids docosahexaenoic acid (m-DHA) and eicosapentanoic acid (m-EPA). Here we tested the hypothesis that m-DHA and m-EPA may be more cytotoxic than the nonmethylated DHA and EPA in inducing apoptosis in breast cancer cells. Data from such studies will provide a foundation for manufacturing supplements that might improve cancer therapy. (ii) We also investigated the role of omega-fatty acids at a fundamental molecular level. Previous biophysical measurements *in vitro* have demonstrated that omega-3 fatty acids influence the organization of the plasma membrane by phase separating from lipid raft molecules, which may have profound implications for signaling and apoptosis. Lipid rafts are sphingolipid-cholesterol-rich lipid microdomains that accumulate predominantly saturated acyl chains and generally serve as platforms for cellular signaling events. In the present study, we tested the role of omega-3s in phase separation from lipid rafts *in vivo*. We examined the localization of various fatty acids into raft vs. non-raft domains using the biochemical detergent extraction method in breast cancer cells. These findings have implications for the role of omega-3 fatty acids in influencing the architecture of the plasma membrane and thereby its function.

METHODS: (i) MDA-231 breast cancer cells were cultured in T75 flasks and then incubated for 24 hr in 96-well plates with increasing concentrations (0-200μM) of various fatty acids including EPA, m-EPA, DHA, or m-DHA. These plates were then subjected to cell viability measurements using WST-1 or LDH assays. (ii) To analyze localization of fatty acids into raft vs. non-raft domains, a cold detergent extraction method was used. Cultured MDA-231 cells were treated with either 25 μM EPA, DHA, arachidonic acid (AA), oleic acid (OA), palmitic acid (PA), cholesterol (CHOL), or retinol spiked with trace amounts of the appropriate radioactive lipid for 24 hr. Cells were scraped and incubated in the presence of 1% Triton X-100 and subsequently placed in a sucrose gradient consisting of 5-35% sucrose in buffer. Sucrose gradients were then subjected to centrifugation at 200,000 *g* for 20 hr at 4 °C. Fractions of 1 mL each were collected, and 50 μL of each isolated sucrose gradient fraction containing trace amount of radioactivity was placed in a scintillation vial and counted using a Beckman scintillation counter (LS 6000 IC).

RESULTS: (i) Cell viability results indicate that omega-3 fatty acids in their free form seem to be more cytotoxic for breast cancer cells than the methyl or ethyl form, contrary to our hypothesis. (ii) Our detergent extraction studies established that our controls (PA and CHOL) were localized to raft fractions (fractions 4-6) in breast cancer cells. In agreement with our hypothesis, EPA, retinol, and DHA were localized predominantly (~90%) in the non-raft regions (fractions 8-12). AA and OA were largely found in the non-raft phase but contained a higher portion of radioactivity in raft fractions 4-6 than either DHA or EPA, consistent with predictions from previous *in vitro* biophysical studies.

CONCLUSIONS: It appears that the esterified omega-3 fatty acids do not seem to be the rationale behind greater potency of certain supplements. This suggests that other components of the supplements may be responsible for apoptosis. The detergent extraction experiments were useful in determining how various fatty acids might localize in the plasma membrane, thus promoting phase separations into raft and non-raft domains. This may have implications for protein mediated signaling events that display affinities for raft and non-raft domains.

Laura's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the Department of the Army Medical Research and Material Command.

Laura will begin her first year of medical school this August at the Indiana University School of Medicine here in Indianapolis. In May, Laura graduated from The University of Notre Dame as a Spanish and Science Preprofessional major. She hopes to combine her love for Spanish with her career as a physician. Her Spanish mentors at Methodist Research Institute include the group collectively known as the "Spanish Mafia" as well as her college professors. She would like to give a special thank you to Rafat Siddiqui for his support of her work as well as to Karen Spear and Dr. Zaloga for their involvement in the program. Furthermore, Laura thanks her wonderful parents who have always taken an interest in her education (even though they are not present today). Most importantly, Laura is forever indebted to Methodist Research Institute not simply for giving her this wonderful research opportunity but also for introducing her to Raz, her boyfriend of one year.

2004 PARTICIPANT ABSTRACTS

Chelimo Yego
Eastern University

Preceptors: Carlos Labarrere, MD, and Miguel Ortiz, DVM
Experimental Pathology Laboratory, Methodist Research Institute

EFFECTS OF CRP AND CRP PEPTIDE (RS 83277) ON ACTIVATION OF NFκB AND UPREGULATION OF uPA AND uPAR

Introduction: C-reactive protein (CRP) is an acute phase protein released in the liver in response to various inflammatory cytokines. CRP binds to damaged cellular tissues and components, serving as an opsonin that prepares debris for phagocytosis and clearance by macrophages and leucocytes; thus, levels of CRP intensify or diminish rapidly with the increase or decrease in infections, inflammation, and trauma. The precise biological functions of CRP and its correlation to cancer and cancer progression are not clear. However, various CRP properties are consistent with its binding to various pathogenic microorganisms and damaged cells to mediate phagocytosis and activation of classical complement pathways—a group of three biochemical pathways that contribute to body defense. CRP peptide RS 83277 is a synthetic peptide derived from the amino acid site 175-184 of CRP. Various studies have shown that the peptide has antitumor effects through the activation of human monocytes and alveolar macrophages. We hypothesized that the CRP peptide RS 83277 increases breast cancer cell growth *(in vitro)* through activation of transcription factor nuclear factor-kappa B (NF*k*B) and subsequent upregulation of uPA and its receptor uPAR, respectively.

Methods: A highly invasive breast cancer cell line (MDA-MB-231) was grown in media completed with fetal bovine serum until the cells were approximately 90% confluent. Prior to treatment, cells were starved by replacing the complete media with serum-free media. Cells were then treated by supplementing them with 0 μg/ml CPR, 10 μg/ml CPR, 50 μg/ml CRP, 100 μg/ml CRP, 0 μg/ml peptide, 10 μg/ml peptide, 50 μg/ml peptide ,100 μg/ml peptide, 0 μg/ml CPR + peptide, 10 μg/ml CPR + peptide, 50 μg/ml CPR + peptid, and 100 μg/ml CRP + peptide and thereafter incubated for 4 hours. Western blots were used for the analysis of uPA and uPAR levels using uPA and uPAR antibodies for both the treated cells and media concentrated through centrifugation filtration. Cell extracts from the treated cells were used to perform an enzyme-linked immunosorbent assay, which determines the activation of NF*k*B. The Alexa Fluor 488 goat-anti-mouse IgG antibody, cytospin and immunocytochemistry was used to localize the CRP and peptide proteins.

Results: Expected results would show that the cells treated with 10 μg/ml CPR, 50 μg/ml 100 μg/ml CRP, 10 μg/ml peptide, 50 μg/ml peptide, 100 μg/ml peptide, 10 μg/ml CPR + peptide, 50 μg/ml CPR + peptide, and 100 μg/ml CRP+ peptide would have an increase in the amount of uPA observed on Western blots, cytospin and immunocytochemistry, as compared to the cells not treated with either CRP or peptide. The cells treated with greater concentrations of CRP, peptide, and both CRP and peptide will have higher levels of uPA. Cells treated with the peptide as well as CPR+ peptide are expected to have an equal amount of uPA produced as cells treated with CRP and this would be indicated by similar sizes in the bands of the Western blots.

Cells treated with CRP, CRP + peptide ,and the peptide alone are expected to have higher levels of NF*k*B activation when compared to the control cells. The cells with greater concentrations of the incubated proteins will have higher levels of NFkB activation. Cells treated with the peptide may increase the level of NFkB, uPA, and uPAR.

Conclusion: C-reactive protein and CRP peptide (RS 83277) may promote the growth of breast cancer cells as secretion of urokinase plasminogen activator is increased. Activation of the nuclear factor kappa B will consequently promote the upregulation of uPA and uPAR, resulting in increased migration, adhesion, and invasion of cells.

Chelimo's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the Department of the Army Medical Research and Materiel Command.

Chelimo graduated from Eastern University in May 2004. She is currently planning on working in the research field for a year before attending dental school.

2004 PARTICIPANT ABSTRACTS

Heidi R. Yount
Wright State University School of Medicine

Preceptor: Rafat Siddiqui, PhD
Cellular Biochemistry Lab, Methodist Research Institute

ROLE OF THE CALPAIN PROTEOLYTIC PATHWAY IN CANCER-INDUCED CACHEXIA

Introduction:
The calpain proteolytic pathway is one of three proteolytic pathways in the body and is responsible for degrading myofibers into myofilaments. The calpain pathway is of interest to study because it functions as the initial step for muscle catabolism, such as in cancer-induced cachexia. Recently, it was discovered that the receptor for the epidermal growth factor (EGF) activates a specific isoform of the calpain protein, calpain II or m-calpain[1]. The purpose of this study was to determine if two omega-3 fatty acids, DHA and EPA, inhibit the EGF receptor and activation of the calpain pathway. This project also investigated the role, if any, of advanced glycation end products (AGE's) in proteolysis. AGE's activate several signal transduction pathways, including $p21^{ras}$, erk ½ kinases, and NF-κB, which lead to inflammation[2], and perhaps, proteolysis.

Methods:
To study the activation of the calpain pathway, several experiments were conducted. First, differentiated skeletal muscle cells were used in tyrosine release assays to measure the amount of proteolysis after specific treatments. Proteolysis was measured through the release of tritiated tyrosine. Next, proteins from MDA cells treated with DHA and EPA were separated by gel electrophoresis and analyzed with western blots. The antibodies used with the western blots were the EGF receptor antibody, the AKT/phospho-AKT antibodies, and the phospho-Map kinase antibody. Finally, gelatin zymography was performed to determine if any m-Calpain was present in the MDA lysates. Tyrosine release assays were also performed with glycated albumin and media supplemented with varying concentrations of glucose to study the effects of AGE's on proteolysis.

Results:
Tyrosine release assays show that 50 mM DHA reduced proteolysis in skeletal muscle cells treated with growth media alone and also with cells treated with growth media from MDA-MB-231 cancer cells. 50 mM EPA showed no effect on proteolysis of the skeletal muscle cells.

The gelatin zymography studies are not complete at this time, but the expected results are a decrease in the amount of calpain protease activity in the cells treated with DHA.

Western blot analysis of the MDA lysates treated with DHA and EPA does not appear to have any effect on the activation of the EGF receptor, nor AKT activation, but the results are inconclusive.

Tyrosine release assays using the glucose supplemented media showed a slight decrease in proteolysis at higher glucose concentrations, while the cells treated with glycated albumin show no increase in proteolysis compared to the controls.

Conclusions:
DHA exhibits a two-fold decrease in proteolysis of skeletal muscle cells when treated with media alone and a three-fold decrease in cells treated with MDA media containing proteolysis inducing factor. Although the calpain zymography results are not finalized at this time, these results support the conclusion that DHA inhibits proteolysis by modulating the calpain proteases.

None of the western blot results at this point appear to show any effect on the activation of the EGF receptor, or AKT activation, but the results are inconclusive, as are the results linking a high glucose level in the cell to increases in advanced glycosylated end products and proteolysis through an AGE receptor.

References:

1. Glading, A., P. Chang, D. Lauffenburger, and A. Wells. (2000). *The Journal of Biological Chemistry* **275**, 2390-2398.
2. Basta, G., G. Lazzerini, M. Massaro, T. Simoncini, P. Tanganelli, C. Fu, T. Kislinger, D. Stern, A. Schmidt, and R. de Caterina. (2002). *Circulation* **105**, 816-822.

Heidi's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the Department of the Army Medical Research and Material Command.

Heidi graduated from Ohio Northern University this spring with a bachelor's degree in Biochemistry and is now a medical student at Wright State University in Dayton Ohio.

Methodist Research Institute

Clarian Health
Methodist·IU·Riley

Summer Student Research Program's

PRESENTATION DAY 2004

PRESENTATIONS

Inhibition of Cancer Growth with *Ganoderma lucidum.*

By: Brian Bock

Dr. Daniel Sliva

Cancer Research Laboratory

Methodist Research Institute

Brian's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the Department of the Army Medical Research and Materiel Command.

Inhibition of Cancer Growth with *Ganoderma lucidum.*

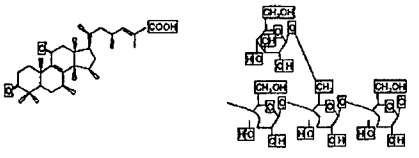
By: Brian Bock
Dr. Daniel Sliva
Cancer Research Laboratory
Methodist Research Institute

Brian's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the Department of the Army Medical Research and Materiel Command.

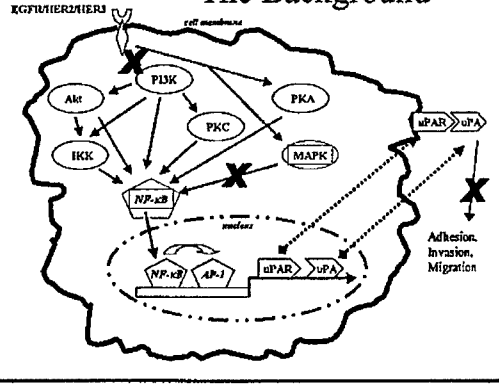
Ganoderma lucidum

ReishiMax GLp™

6% triterpenes & 13.5% polysaccharides



The Background



Hypothesis

- *Ganoderma lucidum* (*G. l.*) inhibits the growth of breast cancer cells.
  - Through the Epidermal Growth Factor Receptor (EGFR) family signaling.
  - Through breast cancer tumors in xenotransplant model.
- Experimental Goals:
  - To determine the mechanism for cancer cell growth.
  - Inhibit the growth of the cancer cells.
  - Determine specific inhibitors for cancer cell growth.

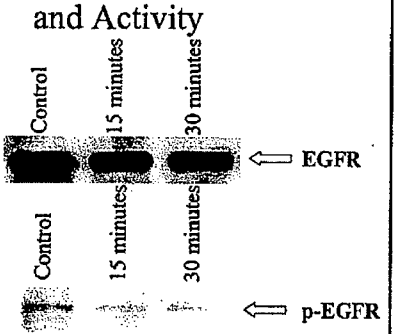
Mechanism of Tumor Growth Suppression In-vitro

- Treated MDA-MB-231 and MCF-7 cells with *G. l.* for different times (15, 30, 60, 120, and 240 minutes).
- Total proteins were extracted and the expression of EGFRs and phophorylated EGFRs were evaluated.

EGFR Family Expression

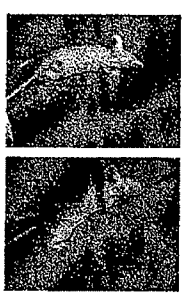
- EGFR Family includes tyrosine kinase receptors EGFR/ErbB1/HER1, ErbB2/HER2, ErbB3/HER3.
- The biological effects of those receptors are exerted through the interaction with a specific ligand, which results in the phosphorylation of EGFR family.

Western Blots of EGFR Expression and Activity

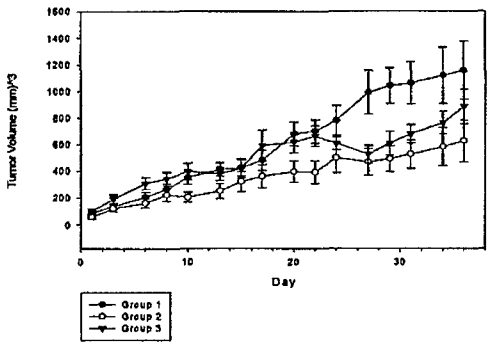


Suppression of Tumor Growth In-vivo.

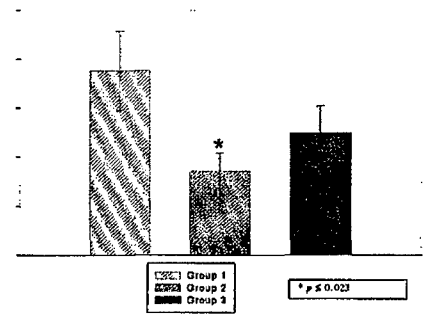
- Nude Mice were used injected subcutaneously with MDA-MB-231 breast cancer cells.
- The Mice were then randomized into three experimental groups.
- Group 1 received a placebo, group 2 100 mg/kg *G. l.*, and group 3 500mg/kg *G. l.*
- Tumor growth on the mice was measured three days a week for 5 weeks.



Average Group Tumor Volume



Final Tumor Weight



Conclusions

- *G. l.* inhibits the phosphorylation of the EGF receptor in MDA-MB-231.
- Western Blots are so much fun and can be done very quickly.
- The 100 mg/kg *G.l.* dose in mice inhibits tumor growth.
  - 500 mg/kg *G.l.* dose may be counterproductive.

Further Research

- Determine *G.l.* effect in EGFR treated MDA-MB-231 and MCF-7 Breast Cancer cells.
- Study EGFR Family expression with immunoprecipitation.
- Start a chemopreventive study on mice.
- Adjunct therapy in mice with *G.l.* and Tamoxifen.

Acknowledgements

Daniel Sliva, PhD and the Cancer Research Lab
Gwenaelle Stanley, Veronika Slivova,
David Jiang, and Colin Terry.

What Really Happened in Group 3.

Effect of Kinase Inhibitors on ERK Activity and Apoptosis in Breast Cancer Cells

Jennifer Griffith

Preceptor: Tom Kovala, PhD

Acknowledgements: George Boguslawski, PhD

Pat McGlynn

Kevin Harvey

Jennifer Griffith's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the Department of the Army Medical Research and Materiel Command.

Effect of Kinase Inhibitors on ERK Activity and Apoptosis in Breast Cancer Cells

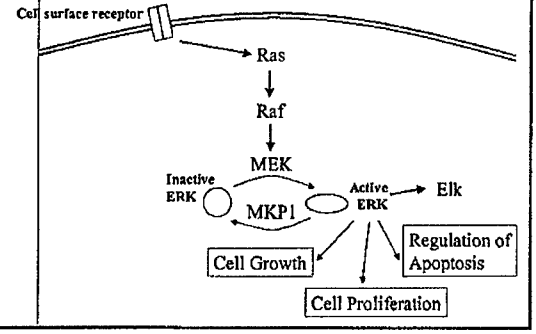
Jennifer Griffith

Preceptor: Tom Kovala, PhD

Acknowledgements: George Bogusławski, PhD
Pat McGlynn
Kevin Harvey

Jennifer Griffith's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the Department of the Army Medical Research and Materiel Command.

MAP Kinase Pathway

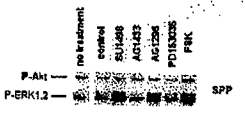


Clinical Relevance

- Increased activation of MAPK pathway in breast cancer cells
  - Highly invasive cell lines (MDA-MB-231 & MDA-MB-468) -- constitutively activated ERK
  - Less invasive cell line (MCF-7) & non-tumor cell line (MCF-10A) -- normal regulation of ERK
- Treat cancer by blocking pathway

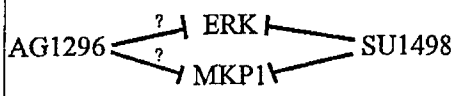
Previous Findings

- SU1498 – inhibitor of vascular endothelial growth factor (VEGF) receptor 2
- Sensitization of breast cancer cells to apoptosis induced by serum starvation
- Build-up of activated ERK in HUVECs



Mechanism of SU1498 Inhibition

- Blocks dephosphorylation of ERK
- Directly inhibits ERK activity



- AG1296 – inhibitor of platelet-derived growth factor (PDGF) receptor

Hypothesis

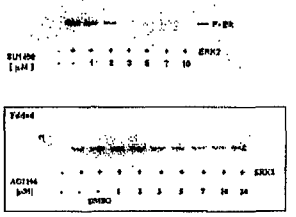
- AG1296 directly inhibits ERK activity and sensitizes breast cancer cells to apoptosis induced by serum starvation.

Methods - ERK Activity Assays

- Reaction samples contain activated ERK and an Elk-1 fusion protein substrate
- Appropriate concentration of inhibitor added to reaction
- SDS-PAGE and western blotting allow detection of phosphorylated Elk-1 product

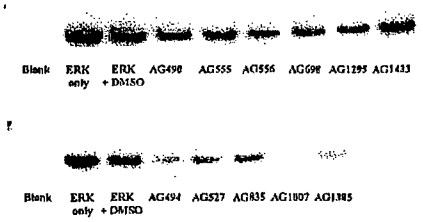
Results - ERK Activity Assays

- SU1498 has dose dependent effect
- AG1296 had no direct effect on ERK



Results - Inhibitor Screen

Compounds structurally similar to either SU1498 or AG1296



Results - ERK Assays cont'd

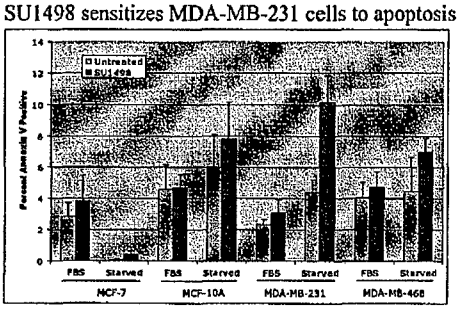
- AG1007 has dose-dependent effect
- Complete ERK inhibition occurs between 1 and 5 μM

Methods - Apoptosis Assays

- Confluent cells incubated for 24 h
  - +/- serum
  - +/- inhibitor
- Cells harvested and collected
- Annexin V fluorescent reagent binds to apoptotic cells
- Analysis performed using flow cytometry

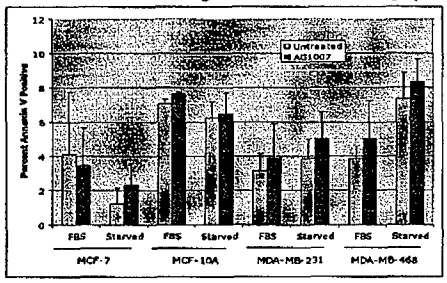
Results - Apoptosis Assays

SU1498 sensitizes MDA-MB-231 cells to apoptosis



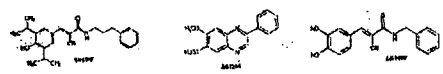
Results - Apoptosis Assays cont'd

AG1007 induces no significant increase in apoptosis



Discussion - ERK Assays

- AG1296 does not affect ERK kinase activity; not a probable candidate to affect apoptosis
- Only AG1007 inhibited ERK at significant levels
- Structural differences may indicate different mechanisms of ERK inhibition



Discussion - Apoptosis Assays

- SU1498 has no effect on apoptosis in the normal and less invasive cell lines
- Compound induces apoptosis in serum-starved highly invasive cancer cells, but not uniformly
- No such effect on apoptosis seen upon treatment with AG1007

Conclusion

- Accumulation of hyperphosphorylated ERK that occurs upon treatment with AG1296 must be due to inhibition of ERK phosphatase activity
- AG1007 directly inhibits ERK kinase, but does not affect apoptosis
- SU1498 remains unique in its ability to sensitize breast cancer cells to apoptosis

Omega-3 Fatty Acids in Apoptosis and Membrane Organization of MDA-231 Breast Cancer Cells

Laura Sech

Preceptors: **Rafat Siddiqui**, PhD and Gary Zaloga, MD

Laura's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the Department of the Army Medical Research and Materiel Command

Omega-3 Fatty Acids in Apoptosis and Membrane Organization of MDA-231 Breast Cancer Cells

Laura Sech

Preceptors: Rafat Siddiqui, PhD
and Gary Zaloga, MD

Laura's participation in the Breast Cancer Research Training Program was supported by grant DAMD20-00-1-0159 from the Department of the Army Medical Research and Materiel Command

Why Omega-3?

- Results conclude a heart failure decrease and cancerous tumor reduction
- Increase in apoptosis and decrease in metastasis
- Surge in production of omega-3 supplements in health food stores

Studies involving Omega-3

- Omega-3 supplement analysis (Cellular Biochemistry Lab 2003)
- ***Oleic and Docosahexaenoic Acid Differentially Phase separate from Lipid Raft Molecules: A Comparative NMR, DSC, AFM nad Detergent Extraction Study*** (Shaikh et. al, 2004, *in press*)

Omega-3 Supplement Analysis

- Supplements are not uniform:
  - Molecular Species Analysis(TLC)
  - Lipid Peroxidation
  - Cell viability and Adhesion(Wst-1 and Vitronectin)

Detergent Resistant Membrane Study

- Omega-3 fatty acids may enhance localization of lipids into structures known as "lipid-rafts."
- Such localization may be responsible for their ability to affect cellular activity

Hypotheses

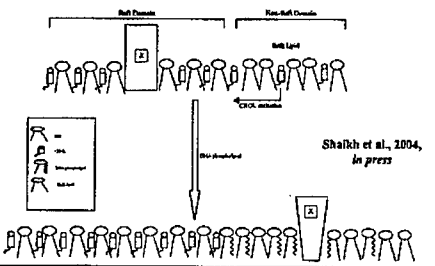
1) The methylated forms of DHA and EPA are more cytotoxic than their free fatty acid components in inducing apoptosis of MDA-231 breast cancer cells.

EPA 

DHA 

Hypotheses cont.

2) The Omega-3 fatty acids EPA and DHA will phase separate more drastically from lipid raft molecules than other cellular components such as Arachidonic acid, Oleic acid, Palmitic acid, Cholesterol, and Retinol.



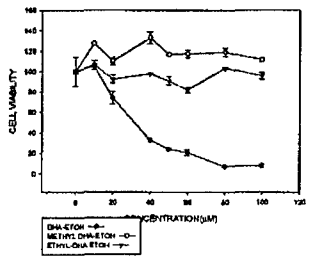
Shaikh et al., 2004, *In press*

Methods

- **WST-1 and LDH cell viability assays**
- **Cultured MDA-231 cells for detergent extraction and treated with either 25uM EPA, DHA, AA, OA, PA, or retinol, along with trace amounts of radioactive lipid**
- **Cell lysates were taken after 24 hours incubation and placed in a sucrose gradient of 5-45% sucrose in buffer**
- **Gradients were placed in an ultracentrifuge for 20 hours at 39,000 rpm**
- **Subsequent 1 mL fractions were collected and analyzed using a Beckman scintillation counter(LS 6000 IC)**

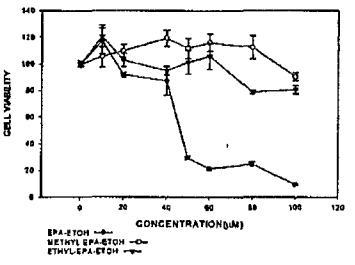
Results WST-1

WST-1 CELL VIABILITY OF MDA CELLS TREATED WITH DHA-ETOH AND DERIVITIVES



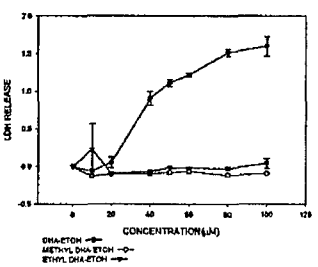
Results WST-1

WST-1 OF MDA CELLS TREATED WITH EPA-ETOH AND DERIVITIVES



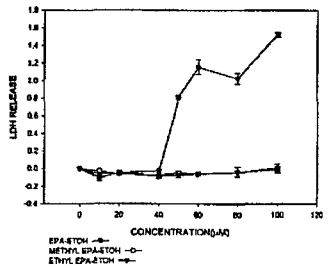
Results LDH

LDH RELEASE OF MDA CELLS TREATED WITH DHA-ETOH AND DERIVITIVES

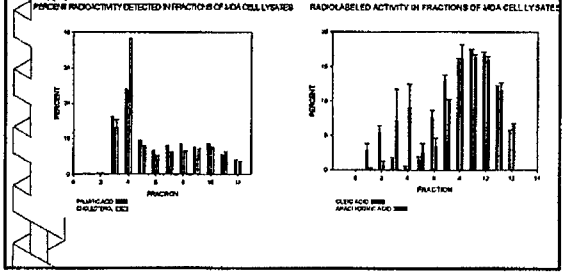


Results LDH

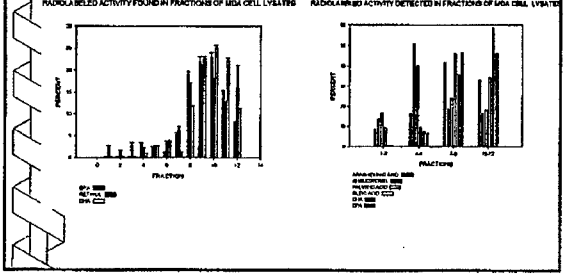
LDH RELEASE OF MDA CELLS TREATED WITH EPA-ETOH AND DERIVITIVES



Results DRM Study



Results DRM Study



Conclusions

- Methylated omega-3 fatty acids do not seem to have an increased cytotoxicity for breast cancer cells.
- Omega-3 fatty acids do localize differently in the plasma membrane than other cell structural components.
- The unique localization of omega-3 fatty acids may have implications for protein mediated signaling events.

Further Research..

- Test other hypotheses to explain the variability of omega-3 supplements
- Do DRM analysis of cells treated with combined EPA/retinol, DHA/alpha-tocopherol, etc. and see if localization differs from independent experimental results
- Perform experiments on Human Mammary Epithelial Cells to see if results differ

Acknowledgements

- Saame Raza Shaikh, PhD. !!!!!
- Alicia Castillo
- Karen Spear, PhD.
- William Stillwell, PhD
- Gulrez Syed, MD

Effects of CRP and CRP Peptide (RS 83277) on Activation of NF-*k*B and Up-regulation of uPA and uPAR in Cancer Cells.

Chelimo Yego
Carlos Labarrere, M.D
Miguel Ortiz, DVM

Chelimo's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the Department of the Army Medical Research and Materiel Command.

Effects of CRP and CRP Peptide (RS 83277) on Activation of NF-*k*B and Up-regulation of uPA and uPAR in Cancer Cells.

Chelimo Yego
Carlos Labarrere, M.D
Miguel Ortiz, DVM

Chelimo's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the Department of the Army Medical Research and Materiel Command.

Introduction:

C-reactive Protein (CRP)

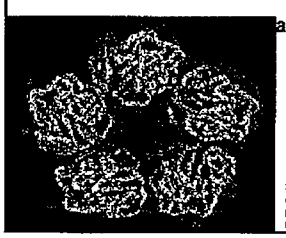
- Is an acute phase protein released in the liver.
- Binds to damaged cellular tissues and components and facilitates phagocytosis .
- Levels of CRP increase with infections, inflammation and trauma.
- Normal serum level is < 1ug/ml.

CRP peptide (RS 83277) is a synthetic peptide derived from the CRP amino acid site 174-185 and could be active on cancer cells.

Actions of CRP:

- Binding to various pathogenic microorganisms and damaged cells to mediate phagocytosis.
- Activation of classical complement pathways.

al function of CRP is not



3D structure of human C-reactive protein.
Greenhough T.J. & co-workers, Keele University, UK.
Image Copyright Keele University
http://us.expasy.org/spotlight/articles/spl8030.html

uPA, uPAR and Cancer:

- Urokinase-type plasminogen activator (uPA) and urokinase-type plasminogen activator Receptor (uPAR) are involved in invasion, migration and adhesion of cancer cells.

- *In vitro* experiments suggest that CRP is involved in the activation of NF- *k*B, which regulates the synthesis of uPA.

Hypothesis:

CRP and CRP peptide (RS 83277) increase breast cancer cell growth (*in vitro*) through activation of transcription factor Nuclear Factor- kappa B (NF-κB) and uPA expression.

Methods:

Cell culture
- Treatment of MDA-MB-231 breast cancer cells with CRP, CRP peptide and CRP+peptide at 0,10, 50 and 100 ug/ml for 4 hours.

Western Blot
- Determine the presence of uPA and uPAR in media and cell lysates.

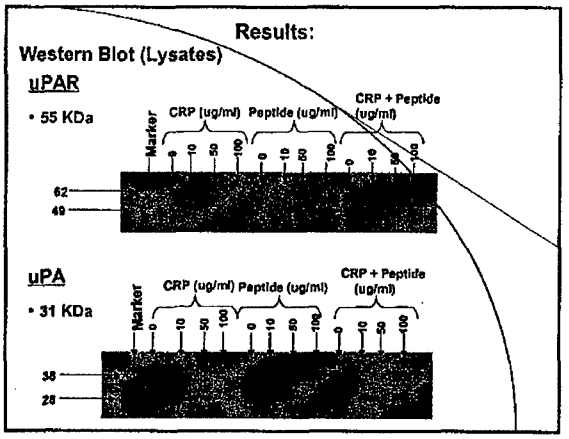
Cytospin and Immunocytochemistry
- Detect presence of uPA and uPAR in the cells.

FACS Analysis (Flow Cytometry)
- Detection of uPAR and uPA on the membranes of treated cancer cells.

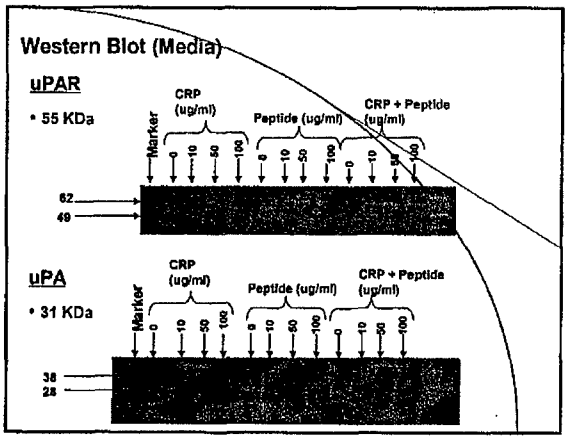
Enzyme Linked Immunosorbent Assay

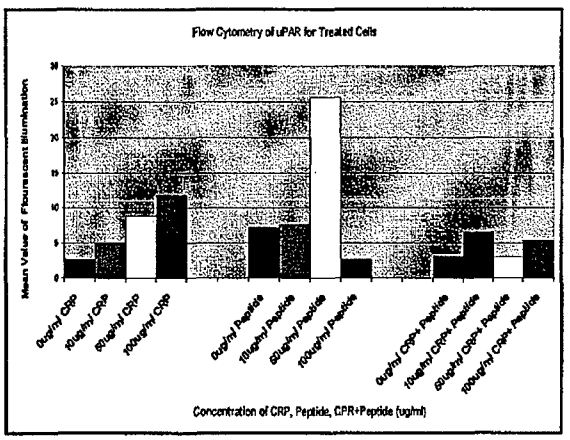
Results:

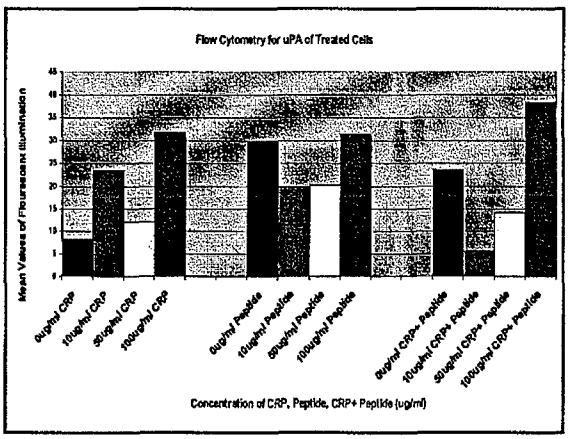
Western Blot (Lysates)

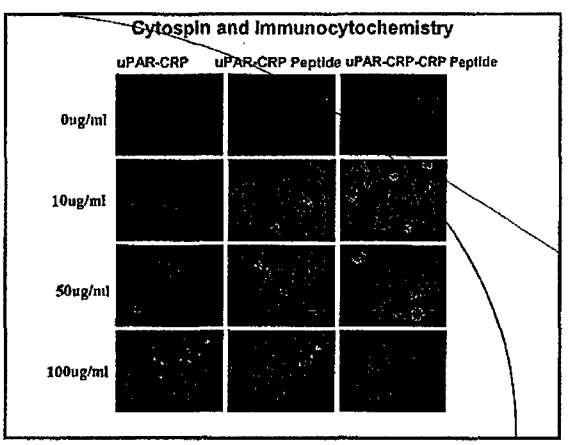


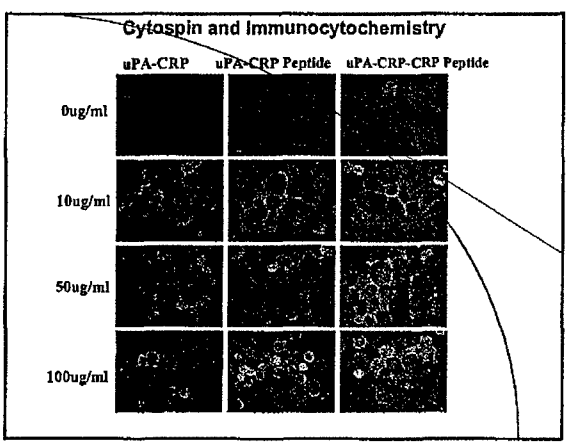
Western Blot (Media)

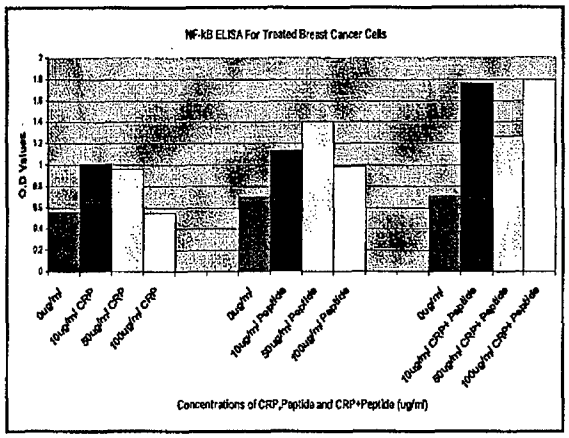


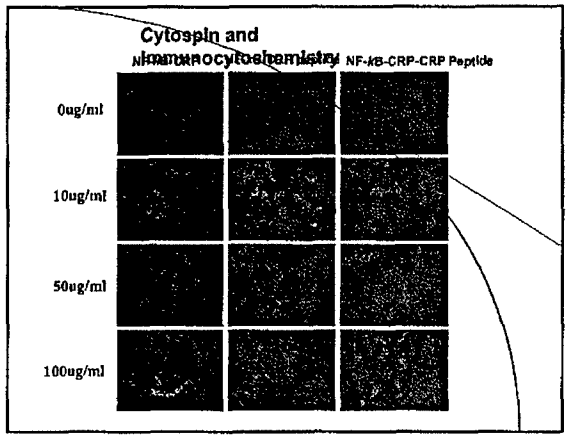

Flow Cytometry of uPAR for Treated Cells
Mean Value of Flouresent Illumination
0ug/ml CRP
10ug/ml CRP
50ug/ml CRP
100ug/ml CRP
0ug/ml Peptide
10ug/ml Peptide
50ug/ml Peptide
100ug/ml Peptide
0ug/ml CRP+ Peptide
10ug/ml CRP+ Peptide
50ug/ml CRP+ Peptide
100ug/ml CRP+ Peptide
Concentration of CRP, Peptide, CPR+Peptide (ug/ml)


Flow Cytometry for uPA of Treated Cells
Mean Values of Flouresent Illumination
0ug/ml CRP
10ug/ml CRP
50ug/ml CRP
100ug/ml CRP
0ug/ml Peptide
10ug/ml Peptide
50ug/ml Peptide
100ug/ml Peptide
0ug/ml CRP+ Peptide
10ug/ml CRP+ Peptide
50ug/ml CRP+ Peptide
100ug/ml CRP+ Peptide
Concentration of CRP, Peptide, CRP+ Peptide (ug/ml)


Cytospin and Immunocytochemistry
uPAR-CRP
uPAR-CRP Peptide
uPAR-CRP-CRP Peptide
0ug/ml
10ug/ml
50ug/ml
100ug/ml


Cytospin and Immunocytochemistry
uPA-CRP
uPA-CRP Peptide
uPA-CRP-CRP Peptide
0ug/ml
10ug/ml
50ug/ml
100ug/ml





Summary of Data:

- CRP levels are associated with increased expression of uPA, uPAR and NF-*k*B on MDA-MB-231 cells.
- CRP peptide seems to be associated with NF-*k*B activation and increased cell-associated uPA and uPAR.

Conclusion:

- Further studies need to be performed in cells and cultured media to quantitatively evaluate possible increased synthesis and release of uPA and uPAR following CRP and CRP-peptide incubation.

Future Studies :

- uPA & uPAR ELISA
  - quantification of uPA & uPAR in cell lysates and cultured media.
- Consider other transcription factors.
  - AP-1

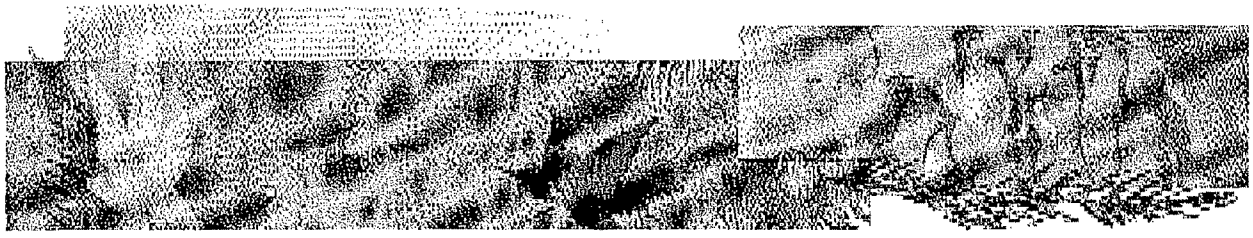
Acknowledgements:

- Roula Antonopoulos
- Marcelo Sosa
- Zach Welch
- Heather Richardson
- Karen Spear
- Chepchumba Yego

Special Thanks to

- Tom Kovala, Ph.D
- Kevin Harvey
- Patrick Mc Glynn

Role of ω-3 Fatty Acids in the Prevention of Muscle Proteolysis



Heidi Yount

July 8, 2004

Heidi's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the Department of the Army Medical Research and Materiel Command.

Role of ω-3 Fatty Acids in the Prevention of Muscle Proteolysis

Heidi Yount

July 8, 2004

Introduction

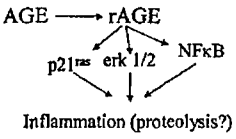
- Cancer cells induce muscle proteolysis by releasing a proteolysis inducing factor
- Proteolysis Inducing Factor increases the activity of the Ubiquitin and Calpain proteolytic pathways
- DHA has been shown to reduce muscle proteolysis in cultured skeletal muscle cells
- m-Calpain is now being measured as a tumor marker

Introduction

- Calpain activation

  EGFR ⟶ ERK ⟶ m-Calpain

- Advanced Glycation End Products



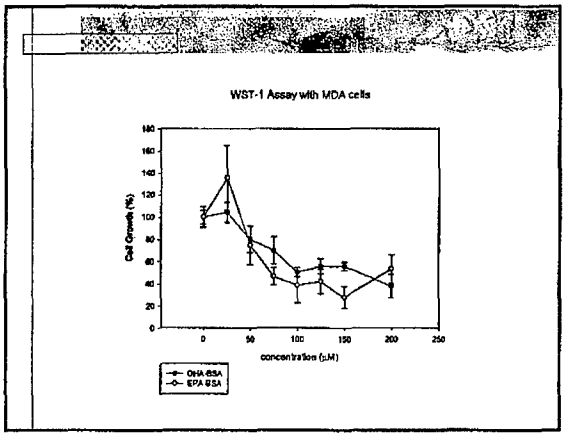
Project Goals

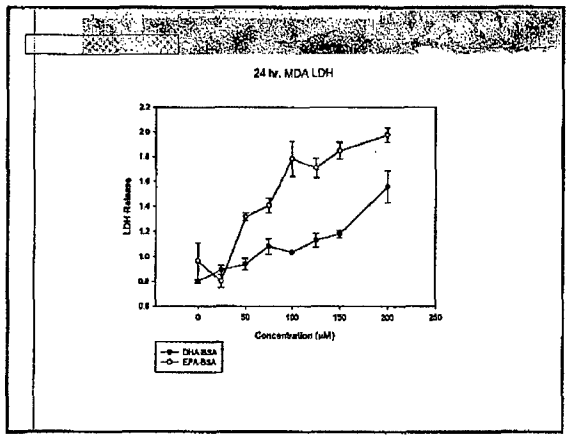
- Determine if glucose and/or glycated albumin induces proteolysis in skeletal muscle cells
- Measure the amount of m-Calpain present in MDA-MB-231 cells compared to mammary epithelial cells
- Test the effect of ω-3 treatment on proteolysis inhibition and m-Calpain activation

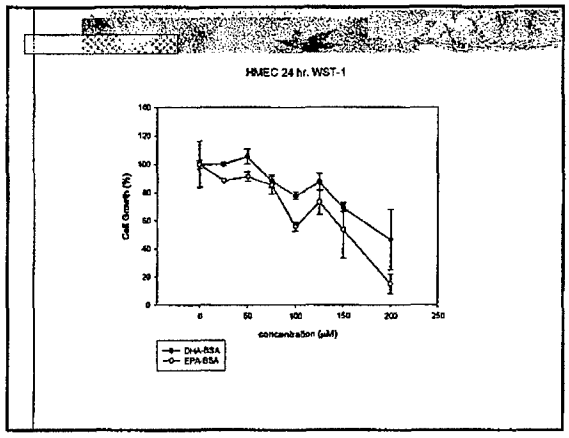
Experiments

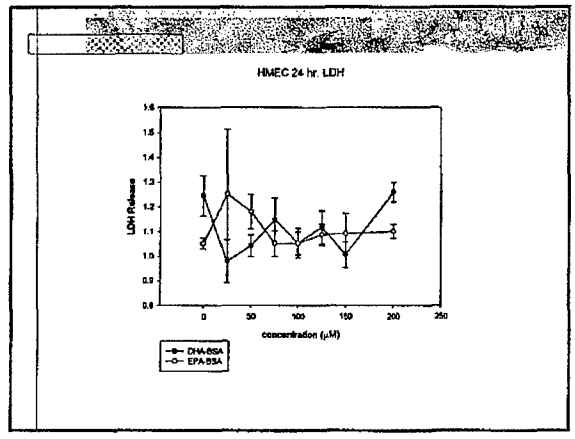
- Cell growth and Cell viability assays
- Tyrosine release assays
- EGFR phosphorylation
- m-Calpain Zymography
- ERK phosphorylation/activation

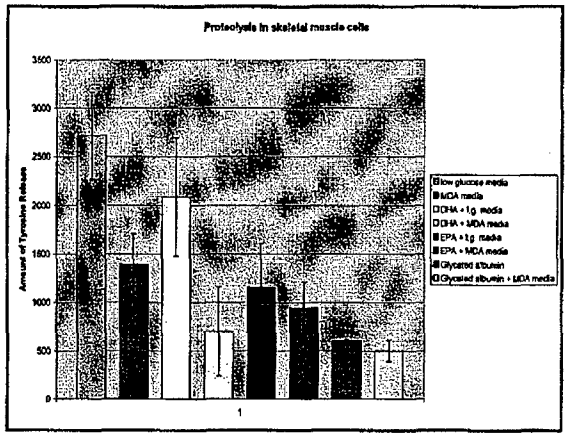
Data

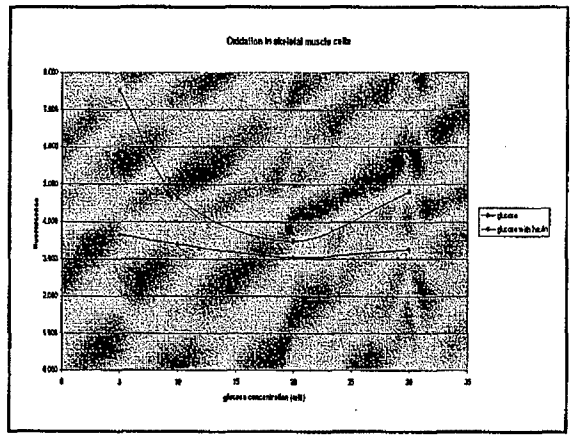





HMEC 24 hr. WST-1
Cell Growth (%)
concentration (μM)
DHA-BSA
EPA-BSA


HMEC 24 hr. LDH
LDH Release
concentration (μM)
DHA-BSA
EPA-BSA


Proteolysis in skeletal muscle cells
Amount of Tyrosine Release
Slow glucose media
MDA media
DHA + 1g media
DHA + MDA media
EPA + 1g media
EPA + MDA media
Glycated albumin
Glycated albumin + MDA media


Oxidation in skeletal muscle cells
glucose
glucose with hcin

Experiments in Progress

- Western blot for EGF receptor in processing
- Mammary epithelial cells are growing again
- Tyrosine release assays continuing
  - Trying serum free MDA media in experiments

INTERNATIONAL
Journal of Cancer

Dr. R.A. Siddiqui
fax +1 317 962 9369
Methodist Research Institute at Clarian Health
Cellular Biochemistry Lab
Indianapolis, IN, USA

18 April 2005; #05-0358-R1

Dear Dr. Siddiqui,

The Editors have now evaluated your revised manuscript "Omega-3 polyunsaturated fatty acids attenuate breast cancer growth through activation of a sphingomyelinase-mediated pathway" and the alterations you have made. I am pleased to inform you that we are satisfied with your additional work and are happy to approve publication of your paper in the International Journal of Cancer for the Fast Track section.

We now ask that you send a Word or RTF file of the final, accepted version of your manuscript, including photographs, figures, and tables (graphic data should be submitted in tif or eps format), to intjcanc@dkfz.de by email. Any necessary permissions, a letter of agreement signed by all authors, and a signed copyright transfer agreement should be sent to the Editorial Office by fax. Please note that in the final version of your manuscript the list of references should be styled according to our referencing guidelines, whereby the references are cited consecutively by number and the list is in order of citation. In the list, all author names should be listed if there are 12 or fewer and if there are 13 or more, list the first 12 and then use "et al." When we have received all these materials, we will send them to our publisher, John Wiley, from whom you will receive your page proofs after about 4-5 weeks. Please consider that the faster you are able to return these proofs, the quicker your article will be published online and appear in the printed journal.

If your paper contains color figures you will receive a Color Cost Reproduction form from the printer. As indicated in the Instructions for Authors, the cost for color is US$500 per page. Please be aware that there is also a charge of US$150 for each page beyond the first eight printed journal pages.

On the cover of every issue of the Journal we are pleased to highlight important work from an article published inside. Our criteria for the image are both high scientific priority and aesthetic appeal. We invite you to submit a suggested cover figure, which will then be considered and judged against the other submitted images. Your selection should be closely related to the subject of your paper but must not necessarily be one of the figures in the paper. If you are interested in contributing a figure, please send a glossy print-out (in the range of 16 cm x 13 cm) to the editorial office at the address below, along with a legend that clearly explains the importance of your figure and its relation to the article being published. We ask that this proposal be sent within one month from the date of this letter.

Thank you for this interesting contribution to the Journal. Please do not hesitate to contact the editorial office if you have any questions about procedure.

Yours sincerely,

Prof. H. zur Hausen
Editor-in-Chief

Editor-in-Chief, Harald zur Hausen
Tel.: 49-6221-424800; Fax 49-6221-424809; E-mail: intjcanc@dkfz.de
Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, Kost. F0820, 69120 Heidelberg . Germany
http://www3.interscience.wiley.com/cgi-bin/jabout/29331/ForAuthors.html

Omega-3 polyunsaturated fatty acids attenuate breast cancer growth through activation of a neutral sphingomyelinase-mediated pathway

Min Wu[1#], Kevin A. Harvey[1], Nargiz Ruzmetov[1], Zachary R.Welch[1], Laura Sech[1], Kim Jackson[2], William Stillwell[2], Gary P. Zaloga[1,3], and Rafat A. Siddiqui[1,2,3*]

[1]Cellular Biochemistry Laboratory, Methodist Research Institute, Clarian Health Partners, Indianapolis; [2]Department of Biology, Indiana University-Purdue University, Indianapolis; and [3]Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana.

Running Title: Fish oil inhibits breast cancer growth

Key Words: Breast Cancer, Docosahexaenoic Acid, Eicosapentaenoic Acid, Fish Oil, Sphingomyelinase, Ceramide

#Present Address: Dept. of Biochemistry & Mol Biology, University of North Dakota, PO Box 9037, Grand Forks, ND58203

* Address for editorial correspondence:
Rafat A. Siddiqui, PhD
Cellular Biochemistry Laboratory
1800 N. Capitol Ave.
Noyes E504
Indianapolis, IN 46202
(317) 962-6941 (tel)
(317) 962-9369 (Fax)
rsiddiqu@clarian.org

ABBREVIATIONS:

DAPI, 4', 6-Diamidino-2-phenylindole; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LOA, linoleic acid; OA, oleic acid; N-SMYase, neutral-sphingomyelinase; p21, waf1/cip1; PS, phosphatidylserine

ABSTRACT

The effect of fish oils and their active omega-3 fatty acid constituents, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), were investigated on breast cancer growth. *In Vivo* Experiments: Mice were fed diets that were rich in either omega-3 (fish oil) or omega-6-rich (corn oil) fatty acids. Three weeks after implantation of MDA-MB-231 breast cancer cells, the tumor volume and weight was significantly lower ($P<0.05$) for mice fed the omega-3 diets compared to those fed the omega-6 diets. Dietary fish oil also caused a 40% ($P<0.05$) increase in neutral sphingomyelinase (N-SMYase) activity in the tumors. The tumor tissues from Fish Oil–fed animals expressed elevated p21 (waf1/cip1) mRNA, whereas tumor tissues from Corn Oil–fed animals exhibited undetectable levels of p21 expression. *In Vitro* Experiments: At concentrations as low as 25 μM, DHA- and EPA- inhibited the growth of cultured MDA-MB-231 cells in a dose-dependent manner by up to 20-25% ($P<0.05$). N-SMYase activity was also increased by 30-40% ($P<0.05$) in the DHA or EPA treated cells in which an increase in ceramide formation was observed. DHA and EPA were both observed to enhance membrane bleb formation and also to induce the expression of p21. Omega-3 fatty acids-induced bleb formation and p21 expression were inhibited by the N-SMYase inhibitor GW4869, which also inhibited apoptosis by ~40% ($P<0.05$). The results suggest that inhibition of breast cancer growth in nude mice by dietary fish oil and inhibition of breast cancer cell growth in culture by treatment with DHA and EPA is mediated by activation of N-SMYase.

INTRODUCTION

The oils of certain cold-water fish have a well-documented role in inhibiting or preventing cancer. Epidemiological evidence strongly links fish oil with low incidences of several cancers. [1-4] The anticancer properties of fish oils have been attributed to the omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Proof of these fatty acids as anticancer agents has been substantiated by dietary studies on many types of animals, including humans and in cultured cells. [5-14] A number of reports have indicated that DHA's anticancer properties are not directly due to cytotoxicity, but rather to the fatty acid's ability to induce apoptosis. [15-18] However, the molecular mechanism for the anticancer actions of omega-3 fatty acids remains unknown. Understanding the mechanistic effects of omega-3 lipids may aid in the development of new cancer therapies.

Numerous studies, including our own, [5, 19-24] have linked fish oil to induction of apoptosis. We found that DHA activates sphingomyelinase (SMYase) activity in the plasma membrane of Jurkat leukemic cells, increasing ceramide levels. [23] SMYase is an enzyme that catalyzes the hydrolysis of sphingomyelin (SM) to ceramide. A variety of studies have shown that ceramide is ubiquitously produced during cellular stress and is associated with apoptosis. [25, 26] To date, at least seven classes of mammalian SMYases have been described, differing in subcellular location, pH optimums, cation dependence, and roles in cell regulation. [27-29] However, only two forms of SMYases, distinguishable by their pH optima, are capable of initiating signal transduction. [30] The acid SMYase (pH optimum 4.5-5.0) is a cellular glycoprotein located in the acidic lysosomal compartment where it contributes to lysosomal SM turnover. [31] The neutral SMYase (N-SMYase; pH optimum 7.4) is a plasma membrane-bound enzyme, [32, 33] that has been implicated in mediating apoptosis in cells exposed to stressing agents. Substantial amounts of N-SMYase are proposed to reside in "lipid rafts".[34] Therefore, factors influencing the lipid composition of membranes can influence the activity and distribution of N-SMYase in "lipid rafts". Recently we have demonstrated that DHA may alter lipid raft formation[35] and induce SMYase activity, leading to cell-cycle arrest in leukemic cells. [23] Furthermore, treating cells with synthetic short-chain ceramide has been shown to induce cell-cycle arrest and apoptosis.[36] Ceramide levels also change during progression through the cell cycle [37] and have been shown to enhance expression of p21 (waf1/cip1) [38], a cellular inhibitor of cdk2 kinase that is involved in cell-cycle arrest *via* hypophosphorylation of retinoblastoma protein (pRb). [39] We have previously show that DHA-induced ceramide may regulate phosphorylation of pRb by inhibition of cyclin A/cdk-2

activities via increased expression of p21.[23] In the project described below, we studied growth inhibition of breast cell xenografts by dietary fish oil in nude mice and we investigated the cellular effects of DHA and EPA in cultured breast cancer cells. Our data demonstrate that the long-chain omega-3 fatty acids inhibit breast cancer growth by activating N-SMYase, thereby generating ceramide.

EXPERIMENTAL

Materials: Human breast cancer MDA-MB-231 cells were obtained from ATCC (American Type Culture Collection, Manassas, VA). Nude mice (nu/nu) were purchased from Charles River Laboratories, Inc., (Wilmington, MA). Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin, and glutamine were from Invitrogen Corporation (Grand Island, NY). Fetal bovine serum was from BioWhittaker (Walkersville, MD). Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), oleic acid (OA), linoleic acid (LOA), and fatty acid standards for gas chromatography (GC) were from Nu-Check Prep, Inc. (Elysian, MN). Annexin V staining, cell death detection ELISA, WST-1 assay, and the lactate dehydrogenase kits were purchased from Roche Biochemicals (Indianapolis, IN). N-SMYase inhibitor, GW4869 was from Calbiochem, San Diego, CA. Hanks Balanced Salt Solution (HBSS), the fish oil (Manhaden), and all other reagents and chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Animal Studies: Nude mice (nu/nu; Charles River Laboratories, Inc., Wilmington, MA) were fed *ad libitum* corn oil (omega-6/omega-3 ratio of 72:1), balanced corn oil/fish oil (omega-6/omega-3 ratio of 1:1), or fish oil (omega-6/omega-3 ratio of 0.11:1) diets (Research Diets, Inc., New Brunswick, NJ) for 3 wk prior to tumor implantation. Diets contained similar quantities of protein (59% of calories), carbohydrates (20% of calories), lipids (21% of calories), vitamins, and minerals as described in Table 1. They only differed in the types of lipids (i.e., corn and fish oil) and their fatty acids composition is described in Table 2. Tumor xenografts were implanted by injecting subcutaneously 200 µl of MDA-MB-231 cells (1×10^6 cells) on the backs of animals, and the animals were returned to their corresponding diets for another 3 wk. Tumor growth was monitored by measuring length and circumference of tumors by a flexible wire tape as described, [40] and tumor weight was determined at termination of the study after excising the tumor free of connective tissue. The tumor tissues were freeze-clamped in liquid nitrogen for later analysis.

Cell Cultures: MDA-MB-231 breast cancer cells were grown in DMEM media containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at a density of 1×10^6 cells/ml for routine culture. For experimental purposes, cells were cultured at the cell density indicated and treated with fatty acids under serum-free conditions. The fatty acids EPA, DHA, OA, and LOA were stored in ethanol under liquid nitrogen and diluted in ethanol

just prior to use. The final concentration of ethanol (< 0.1%) in the treated cultures did not induce any cytotoxic effects as measured by lactate dehydrogenase release and a WST-1 cell proliferation assay (results not shown).

Cell Growth Assay: The effect of the fatty acids on cell growth was determined using a WST-1 assay as per manufacturer's instructions (Roche Biosciences, Indianapolis, IN).

N-SMYase Assay: Sphingomyelinase activity was measured by an Amplex Red sphingomyelinase assay kit (Molecular Probes, Eugene, OR). Briefly, the frozen tumor tissues were ground under liquid nitrogen and then homogenized in a reaction buffer containing 100 mM Tris-HCl (pH 7.5), 50 mM $MgCl_2$, and 0.1% Triton X-100. For measuring sphingomyelinase activities in MDA-MB-231 cells, DHA- or EPA-treated cells were lysed in 100 µl of lysis buffer [20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 100 mM NaF, 2 mM Na_3VO_4, 10% v/v glycerol, 1% Nonidet P-40, 2 mM phenylmethanesulfonyl fluoride (PMSF), 1 mg/ml leupeptin, 0.15 units/ml aprotinin, and 2.5 mM DIFP] for 10 min on ice. Protein concentrations in tumor homogenates, cell lysates, and isolated membrane fractions (see below) were measured using a bicinchoninic acid (BCA) protein assay system (Pierce, Rockford, IL), and samples were diluted in reaction buffer for the assay in the presence of exogenous sphingomyelin (0.25 mM). The released phosphorylcholine from sphingomyelinase activity was measured by the sequential activity of alkaline phosphatase and choline oxidase. The resultant release of H_2O_2 was quantified by measuring fluorescence intensities (excitation at 540 nm and emission at 590 nm) after reaction with the Amplex Red reagent as described in the manufacturer's protocol. Amount of sample protein (~10 µg) resulted in a linear fluorescence intensity between ~$1/5^{th}$ to $1/10^{th}$ of the positive control.

Analysis of p21 mRNA expression: RNA from tumor tissues or cultured cells was extracted by a RNA assay kit (Qiagen, Valencia, CA). The amount of RNA in an aqueous solution was determined by absorbance at 260 nm. Semi-quantitative RT-PCR was performed to determine the mRNA levels of p21 and GAPDH (loading control) using the Titan One Tube RT-PCR System (Roche Diagnostics, Indianapolis, IN). The primer sequences 5'CGG-TCC-CGT-GGA-CAG-TGA-GCA-G3', 5'GTC-AGG-CTG-GTC-TGC-CTC-CG3' were used for p21. The thermocycling parameters were composed of an initial cycle at 50°C for 30 min for reverse transcription of RNA into cDNA. The subsequent DNA amplification was performed

with a thermocycling reaction consisting of 95°C for 180s followed by 30 cycles at 95°C for 60s, 55°C for 60s, and 72°C for 60s.

Western blot analysis: Following treatment with DHA or EPA, cell lysates were separated by SDS PAGE (10%), and then electro-blotted onto presoaked Immobilon-P membranes (Millipore, Bedford, MA) as described previously.[23] The membranes were blocked in 5% dry milk in TBS-T solution (50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 2 hr at room temperature. The blot was incubated with monoclonal anti-p21 or monoclonal anti-GAPDH antibodies (Santa Cruz Biotech, Santa Cruz, CA; 1:1000) at 4°C overnight and detected using secondary anti-rabbit peroxidase-conjugated antibodies (Amersham Pharmacia Biotech, Buckinghamshire, UK; 1:2000 in TTBS). The bands were detected using a chemiluminescence detection kit (Pierce, Rockford, IL).

Immunohistochemistry: Ceramide and p21 formation were determined using immunohistochemistry. [41] After incubation with serum-free media containing DHA or EPA, the cells were fixed with 3% paraformaldehyde and then blocked with 1% BSA in PBS. Ceramide was detected using a specific anti-ceramide antibody (Alexis, Carlsbad, CA; clone MID 15B4; 1:200 dilution in blocking buffer) and assayed using an Alexa 488-labelled anti-mouse antibody (Molecular Probes, Eugene, OR; 1:200 dilution in blocking buffer), whereas p21 was detected using anti-p21 (Santa Cruz Biotech, Santa Cruz, CA; 1:200 dilution in blocking buffer) and assayed using an Alexa-546-labelled mouse antibody. Presence of the nuclei was detected by DAPI stain. Cells were examined under a fluorescence microscope and pictures were taken using a MagnaFire digital camera (Optronics, Goleta, CA) for analysis.

Cell Death ELISA: Quantitative analysis of DNA fragmentation was carried out using a histone-based Death ELISA system (Roche, Indianapolis, IN) as per manufacturer's protocol. MDA-MB-231 cells (1 x 10^6/ml) in 6-well plates were incubated with fatty acids for 24 hr and then lysed. The nucleosomes containing fragmented DNA were captured by an immobilized anti-histone antibody. The amounts of DNA fragments were then determined spectrophotometrically using a peroxidase-conjugated anti-DNA antibody.

Annexin V Staining: Externalization of phosphatidylserine was evaluated using an annexin V staining kit (Roche Biochemicals, Indianapolis, IN) as per manufacturer's instruction. This kit can distinguish apoptotic and necrotic (dead) cells by propidium iodide (red) staining and FITC-conjugated annexin V (green) staining, respectively. The cells were visualized using a fluorescence microscope. The total and apoptotic cells were counted and the percentage of cells exhibiting apoptosis was calculated.

Statistics: All experiments were performed at least three times each in triplicate and expressed as mean ± SE. Comparisons were done using a Student's *t* test and one-way ANOVA. Significance was defined as $p<0.05$.

RESULTS

Inhibition of Breast Cancer Tumors by Dietary Fish Oil (*In Vivo* Studies): The effect of dietary fish oil on breast cancer growth was investigated in nude mice. Results demonstrate that mice fed a corn oil diet rich in omega-6 fatty acids exhibited substantial tumor growth (Figure 1a). In contrast, mice fed on the omega-6/omega-3–balanced diet exhibited very reduced cancer growth (Figure 1b). Tumor growth in mice fed the omega-3–rich fish-oil diet had by far the smallest tumors (Figure 1c). Because the tumors were asymmetric, quantification was achieved by measuring tumor volume, surface area, and mass. Data shown in Table 3 indicate that, 3 wk following tumor implantation, tumor volume was approximately 60% lower ($P<0.05$), whereas tumor surface area and weight were approximately 30-40% lower ($p<0.05$) in mice maintained on the balanced diet (omega-3/omega-6, 1:1) compared to mice on the corn oil diet (omega-6 enriched). The largest reduction in tumor volume was noted for the fish-oil diet (omega-3 enriched), where tumor volume was approximately 75% lower ($p<0.05$), and tumor surface area and weight were approximately 50% lower ($P<0.05$) compared to animals maintained on corn oil diets.

Tumor tissue isolated from mice maintained on each of the three diets was then analyzed for N-SMYase activity. Data in Figure 2a show that N-SMYase activity was higher by approximately 40% ($P<0.05$) in the tissues from mice raised on the omega-3–containing diets (fish oil and balanced fish/corn oil) compared to those on the high omega-6 corn oil diet.

Tumor tissues were further analyzed for p21 expression using semi-quantitative RT-PCR. Results presented in Figure 2b demonstrate that expression of p21 mRNA was upregulated by fish oil. mRNA expression of p21 in mice maintained on the fish oil diet was induced, while p21 was not detected in tumor tissues of corn-oil-diet–fed mice.

Effects of Omega-3 Fatty Acids on Breast Cancer Cells in Culture (*In Vitro* Studies): We next evaluated whether the long-chain polyunsaturated omega-3 fatty acids commonly found in fish oils (DHA and EPA) can affect MDA-MB-231 breast cancer cells *in vitro*. The dietary fish oil (Menhaden, Sigma Chemical Co.) employed in these studies was first analyzed for its fatty acid content by gas chromatography. The fish oil was shown to have 150 mg of DHA and 160 mg of EPA per g of oil (Table 2). Both DHA and EPA were then tested on cultured MDA cells where both fatty acids similarly inhibited growth in a dose-dependent manner (Figure 3). At concentrations as low as 25 μM, both fatty acids inhibited cell growth by approximately 25-30% ($P<0.05$), and the inhibitory effects of DHA and EPA progressively increased with increasing concentration to a maximum inhibition at 80 μM by approximately 80% ($P<0.05$) after 24 hr of incubation.

We further tested whether other long-chain unsaturated fatty acids had effects similar to DHA and EPA on breast cancer growth. Results shown in Figure 4 demonstrate that at a concentration where DHA and EPA significantly inhibited MDA growth (25 μM), oleic acid, an omega-9 fatty acid, and linoleic acid, an omega-6 fatty acid, had only a minimal effect on growth. However, a concentration over 100 μM of OA or LA resulted in a similar effect as of DHA or EPA at 25 μM. Therefore at lower concentrations inhibition of cancer cell growth was not a general consequence of any long-chain fatty acid but rather is unique to the long-chain polyunsaturated omega-3 fatty acids whereas at higher concentrations all fatty acids are generally cytotoxic (detergent effect).

We further examined whether growth inhibition of MDA cells was due to induction of apoptosis. Results presented in Figure 5a demonstrate that both DHA and EPA at 25 μM induce apoptosis by 70- 75% ($P<0.05$) as is evident from enhanced DNA fragmentation. Furthermore, N-SMYase activity was increased by 30-40% ($P<0.05$) in cells treated with DHA or EPA compared to those of untreated (control) cells (Figure 5b). The increase in N-SMYase activity induced by the omega-3 fatty acids was further analyzed by assaying

ceramide formation, the product of sphingomyelin hydrolysis. The noticeable generation of ceramide was observed in MDA cells upon DHA or EPA treatment (Figure 6a) compared to that of control cells. We also analyzed the effect of DHA and EPA on p21 expression by both RT-PCR and by Western analysis. Results presented in Figure 6b indicate that expression of p21 protein was increased approximately 2.5-3-fold ($P<0.05$) in DHA- or EPA-treated cells compared to control cells. Similarly, DHA and EPA also increased expression of p21 mRNA in the same cells (Figure 6c).

Effect of Omega-3 Fatty Acids on Membrane Structure: One of the very noticeable effects of DHA on MDA cells was the induction of plasma membrane blebs. Results shown in Figure 7a demonstrate substantial changes in appearance (bleb formation) of the MDA cell surface caused by increasing concentrations (0-100 μM) of DHA. The MDA cells were then stained with annexin V for the presence of externalized phosphatidylserine. Figure 7b shows that cells incubated in 50 μM DHA demonstrated extensive bleb formation, and these blebs appear to have aggregated phosphatidylserine on the surface (the membrane blebs and annexin V are colocalized). Similar effects on membrane bleb formation were also observed upon treatment with EPA (data not shown). In addition, bleb formation appears to be related to the DHA-induced N-SMYase activity reported in Figure 5b. Results presented in Figure 7c indicate that the DHA-induced membrane bleb formation was inhibited by the N-SMYase inhibitor GW4869.

Inhibition of Omega-3 Fatty Acid-Induced p21 Expression and Apoptosis by N-SMYase Inhibitor: The involvement of N-SMYase in DHA- or EPA-induced p21 expression was further investigated using immunohistochemistry. Figure 8a indicates that expression of p21 protein was induced in DHA- and EPA-treated cells as is evident by enhanced fluorescence intensities. This DHA- or EPA-induced expression of p21 was markedly diminished by approximately 50-60% in the presence of N-SMYase inhibitor.

Involvement of N-SMYase in DHA- or EPA-induced apoptosis was also investigated. Data depicted in Figure 8b indicate that in the presence of the N-SMYase inhibitor, DHA- and EPA-induced apoptosis in MDA cells was inhibited by approximately 40-50% ($P<0.05$).

DISCUSSION

Breast cancer is one of the most frequently diagnosed non-skin cancers and the second most common cause of cancer death in women. [42] An estimated 215,990 new cases of breast cancer were expected in 2004. Epidemiological evidence links fish oil consumption (rich in the omega-3 fatty acids DHA and EPA) with a low incidence of several types of cancer. [1-4] The anticancer role of omega-3 fatty acids has also been substantiated with dietary studies using many types of animals (including humans) and numerous different cell lines, including breast cancer. [8, 10, 19, 20] In the study reported here, we investigated the effects of long-chain polyunsaturated omega-3 fatty acids on breast cancer cells both *in vivo* and *in vitro*.

Our *in vivo* studies, shown in Figures 1-2 and Table 3, demonstrate that increasing the ratio of omega-3 to omega-6 fatty acids in the diet inhibits development of transplanted breast cancer cells in nude mice. Similar animal models to study the growth of transplanted breast cancer cells have been widely described.[5] The oils used for preparing the diets were examined for lipid peroxidation products as previously described [24] and tested for apoptosis on breast cancer cells. As previously reported for Jurkat cells, our data indicates that levels of lipid peroxidation products in oils did not correlate with the extent of apoptosis. It therefore appears that the oils but not the oxidized products are responsible for the cytotoxic effects in breast cancer cells. The purpose of this study was to link the omega-3 fatty acids abundant in dietary fish oil (DHA and EPA) to inhibition of breast cancer cell proliferation *in vivo* and to investigate one possible mode of action, namely the effect on N-SMYase.

Our studies were then extended to cultured MDA-MB-231 cells, in which we initially investigated whether the constituents of fish oil—DHA and EPA—could inhibit breast cancer proliferation *in vitro*. Data presented in Figure 3 indicate that both DHA and EPA were equally effective in inhibiting MDA-MB-231 cell growth. Similar inhibition was not observed with either oleic acid, an omega-9 fatty acid that is the most abundant fatty acid in animal tissues, or linoleic acid, an omega-6 fatty acid abundant in common vegetables oils (see Figure 4), indicating that inhibition is not just a general property of all long-chain fatty acids. The data presented in Figure 5a indicate that inhibition of cancer cell growth was likely due to induction of apoptosis by the omega-3 fatty acids.

We further evaluated a possible signaling pathway that may be responsible for regulating tumor growth by omega-3 fatty acids. Our previous studies suggest that DHA inhibits the

growth of Jurkat leukemic cells partially through activation of N-SMYase, increased ceramide formation, and enhanced expression of p21. [23] To link the breast cancer studies to our previous work with Jurkat leukemic cells, we monitored the activity of N-SMYase and p21 expression both *in vivo* (nude mice) and *in vitro* (cultured MDA-MB-231 cells). Results shown in Figure 2 demonstrate that tumors from mice fed a corn oil (omega-6)-based diet have considerably lower N-SMYase activity than mice fed on diets containing significantly higher omega-3 fatty acid levels. Similarly, treatment of cultured MDA-MB-231 cells with DHA or EPA enhanced N-SMYase activity (Figure 5b), resulting in increased ceramide formation (Figure 6a) and enhanced p21 expression as analyzed by Western blot and immuno-histochemical analysis (Figure 6b) and confirmed by RT-PCR (Figure 6c). RT-PCR in the present study was performed as an extra measure to confirm increased expression of p21 in the presence of DHA or EPA. Although products generated after 30 cycles do not indicate a quantitative measure of p21 mRNA expression in these cells, they clearly demonstrates differences between control and treated cells.

Ceramide-induced activation of cellular N-SMYase–mediated signaling pathways in response to stress has been previously reported by Hannun *et al* [43] and Kolesnick *et al.* [44] The N-SMYase pathway is known to be activated by various factors including heat, ischemia/reperfusion, oxidants, tumor necrosis factor-alpha (TNFα, Fas ligand, vitamin D3, IL-1, and γ-interferon and initiates cellular events leading to cell death or apoptosis. [45, 46] Hydrolysis of SM by N-SMYase generates ceramide, a lipid that is regarded as a "universal component of apoptosis".[47-49] About 70% of cellular SM is present in the outer leaflet of plasma membranes, primarily in lipid rafts, where it probably serves to stabilize rafts by interacting with cholesterol and phospholipids containing saturated fatty acids. [50, 51] Several studies suggest that N-SMYase is generally localized in plasma membranes. [32, 52] Activation of cells by TNF has been shown to induce translocation of N-SMYase from detergent-resistant membrane fractions (lipid rafts) to detergent-soluble (nonraft) fractions, resulting in enhanced activity. [34] In a preliminary study (data not shown), we have also demonstrated that DHA treatment decreases N-SMYase activity in the detergent-resistant (raft) fractions, while increasing activity in the detergent-soluble (nonraft) fractions. These results suggest that omega-3 fatty acids induce changes in the plasma membrane composition and structure of MDA-MD-231 cells, affecting N-SMYase activity by either translocating the enzyme from detergent-resistant to detergent-soluble fractions or by directly affecting N-SMYase activity

(conformational change) in these fractions. While interesting, these results need to be verified using specific antibodies against N-SMYase, which are under development in our laboratory.

We have previously demonstrated that DHA causes substantial changes in the domain structure of model membranes and isolated plasma membranes [35] and that incorporation of DHA instigates cellular changes leading to apoptosis. [23] The experiments reported here further suggest that the addition of DHA causes visible changes in the plasma membrane as indicated by the appearance of membrane blebs with externally exposed PS (annexin V binding) (Figures 7a,b). Furthermore, DHA-induced membrane bleb formation and p21 expression was inhibited by the N-SMYase inhibitor GW4869, indicating that ceramide generation is involved in this process. In fact, blebbing and externalization of PS are hallmarks of the execution phase of apoptosis [53] and are believed to be related to ceramide generation. In agreement with these findings, we observed that the N-SMYase inhibitor also reduced DHA- and EPA-induced apoptosis (Figure 8).

In conclusion, the data presented here strongly indicate a relationship between the omega-3 fatty acids DHA and EPA, tumor growth suppression, membrane structure, N-SMYase activity, ceramide formation, p21 expression, and apoptosis. Our results suggest that modulation of the N-SMYase-ceramide pathway represents a potential pathway for treatment of breast cancer.

FIGURE LEGENDS

Figure 1. Fish oil inhibits breast cancer growth in mice. Nude nice were maintained on corn oil (ω-6/ω-3 ratio 72:1), balanced corn/fish oil (ω-6/ω-3 ratio 1:1) or fish oil (ω-6/ω-3 ratio 0.11:1) diets for 3 wk prior to subcutaneous implantation of MDA-MB-231 cells ($1x10^6$ cells). The animals were then further fed on the corresponding diets for another 3 wk. Results are representations from six mice in each group.

Figure 2. Fish oil enhances N-SMYase activity and p21 expression in tumor tissues. Mice implanted with breast cancer cells as described in the legend of Figure 1 were sacrificed and their tumor tissues were isolated. (a) N-SMYase activity was assayed as described in the Experimental section. Results are the mean ± SEM for three experiments. Results were analyzed by Student's t-test and one-way ANOVA. Significant differences compared to the Corn Oil group are reported (*$P<0.05$). (b) For p21 expression, RNA from tumor tissues were extracted and RT-PCR was performed to determine the mRNA levels of p21 and GAPDH (loading control) using the Titan One Tube RT-PCR System as described in the Experimental section. Negative control (-C) was muscle tissue from a normal mouse; positive control (+C) was RNA isolated from HCT101 $p21^+$ cells. Results are shown for three tumor tissues for each group.

Figure 3. Dose dependent effect of omega-3 fatty acids on breast cancer cell growth. Cells ($1 x10^4$ per well) were seeded in a 96-well plate overnight and then treated with varying concentrations of DHA or EPA in serum-free medium for 24 hr. Cell growth was assayed using a WST-1 assay as described in the Experimental section. Results are the mean ± SEM for three experiments. Results were analyzed by Student's t-test and one-way ANOVA. Significant differences compared to the Corn Oil–fed group are reported (*$P<0.05$).

Figure 4. Effect of different fatty acids on cell growth. MDA-MB-231 cells were treated with 25 μM concentrations of DHA, EPA, oleic acid (OA), or linoleic acid (LOA) and cell growth was assayed as described in the legend of Figure 4. Results are the mean ± SEM for three experiments. Results were analyzed by Student's t-test and one-way ANOVA. Significant differences compared to the control are reported (*$P<0.05$).

Figure 5. Effects of omega-3 fatty acids on apoptosis and N-SMYase activity in breast cancer cells. MDA-MB-231 cells (1 x 10^6/ml) were grown in 6-well plates and treated with 25 μM DHA or EPA as described in the legend of Figure 4. Apoptosis (a) was assayed using a cell death ELISA kit, whereas N-SMYase activity (b) was assayed using an Amplex Red kit described in the Experimental section. Results are the mean ± SEM for three experiments. Results were analyzed by Student's t-test and one-way ANOVA. Significant differences compared to the control are reported (*$P<0.05$).

Figure 6: Omega-3 fatty acids induce ceramide formation and p21 expression in breast cancer cells. MDA-MB-231 cells (1 X 10^4/ml grown in 4-well chamber slides or 1 x 10^6/ml grown in T75 flasks) were treated with 25 μM DHA or EPA as described in the legend of Figure 4. Generation of ceramide (a) was determined immunohistochemically using an anti-ceramide antibody (green fluorescence) as described in the Experimental section. Blue DAPI staining was used to visualize nuclei. Expression of p21 protein was analyzed by Western analysis (b) using GAPDH as a loading control as described in the Experimental section, whereas expression of p21 mRNA was analyzed by RT-PCR (c) as described in the legend of Figure 3. Results are representative of three experiments in each section.

Figure 7: Effect of omega-3 fatty acids on membrane bleb formation. MDA-MB-231 cells (1 x 10^4/ml) were grown in 4-well chamber slides and treated with varying concentration of DHA as described in the legend of Figure 2. Cells were observed under a microscope using 400X magnification (a). The blebs were assayed for externalized phosphatidylserine in cells treated with 50 μM DHA by using an annexin V binding kit as described in the Experimental section (b). Inhibition of omega-3 fatty acid-induced membrane bleb formation was observed in the presence or absence of 20 μM GW4869, a N-SMYase inhibitor (c). Cells were observed under the microscope using 20x magnification. Results are representative of three experiments in each section.

Figure 8: Inhibition of p21 expression and apoptosis by the N-SMYase inhibitor. (a) MDA-MB-231 cells (1 x 10^4/ml) were grown in 4-well chamber slides and then treated with 25 μM DHA or EPA for 24 hr in the presence or absence of 20 μM GW4869. Cells were then fixed and expression of p21 was detected using immunohistochemical methods as described in the Experimental section. Expression of p21 was quantified by densitometric

analysis using a KODAK Image Station 2000MM (Eastman Kodak Company, Rochester, NY). (b) MDA-MB-231 cells in 6-well plates were incubated for 24 hr with 25 μM DHA or EPA in the presence or absence of 20 μM GW4869. Plates were centrifuged at 800 x g in a Beckman J series centrifuge to deposit floating cells at the bottom for analysis and the supernatant was carefully removed. Quantitative analysis of apoptosis was performed by using an annexin V staining kit as described in the experimental section. The dead and necrotic cells exhibit red fluorescence, whereas apoptotic cells fluoresce green. The total and apoptotic cells were counted and the percentage of cells exhibiting apoptosis was calculated. Results are the mean ± SEM for three experiments. Results were analyzed by Student's t-test and one-way ANOVA. Significant differences between groups are reported (*$P<0.05$).

ACKNOWLEDGEMENTS

This work was supported by a grant (BC020180) from the U.S. Department of Army Medical Research and Materiel Command. The authors wish to thank Ms. Alicia Castillo for technical support; Ms. Diane Bond and Ms. Nicole Courtney for animal care; Ms. Heather Richardson and Ms. Charlene Shaffer for secretarial assistance; Dr. Karen Spear for editing the manuscript; and Mr. Colin Terry for statistical analysis of the data.

REFERENCES

1. Vogel VG, McPherson RS. Dietary epidemiology of colon cancer. Hematol Oncol Clin North Am 1989;3:35-63.

2. Kaizer L, Boyd NF, Kriukov V, Tritchler D. Fish consumption and breast cancer risk: an ecological study. Nutr Cancer 1989;12:61-68.

3. Schloss I, Kidd MS, Tichelaar HY, Young GO, O'Keefe SJ. Dietary factors associated with a low risk of colon cancer in coloured west coast fishermen. S Afr Med J 1997;87:152-58.

4. Berg JP, Glattre E, Haldorsen T, Hostmark AT, Bay IG, Johansen AF, Jellum E. Longchain serum fatty acids and risk of thyroid cancer: a population- based case-control study in Norway. Cancer Causes Control 1994;5:433-39.

5. Connolly JM, Gilhooly EM, Rose DP. Effects of reduced dietary linoleic acid intake, alone or combined with an algal source of docosahexaenoic acid, on MDA-MB-231 breast cancer cell growth and apoptosis in nude mice. Nutrition & Cancer 1999;35:44-49.

6. Rose DP, Connolly JM. Antiangiogenicity of docosahexaenoic acid and its role in the suppression of breast cancer cell growth in nude mice. International Journal of Oncology 1999;15:1011-15.

7. Ogilvie GK, Fettman MJ, Mallinckrodt CH, Walton JA, Hansen RA, Davenport DJ, Gross KL, Richardson KL, Rogers Q, Hand MS. Effect of fish oil, arginine, and doxorubicin chemotherapy on remission and survival time for dogs with lymphoma: a double-blind, randomized placebo-controlled study. Cancer 2000;88:1916-28.

8. Noguchi M, Minami M, Yagasaki R, Kinoshita K, Earashi M, Kitagawa H, Taniya T, Miyazaki I. Chemoprevention of DMBA-induced mammary carcinogenesis in rats by low-dose EPA and DHA. British Journal of Cancer 1997;75:348-53.

9. Takahashi M, Totsuka Y, Masuda M, Fukuda K, Oguri A, Yazawa K, Sugimura T, Wakabayashi K. Reduction in formation of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced aberrant crypt foci in the rat colon by docosahexaenoic acid (DHA). Carcinogenesis 1997;18:1937-41.

10. Rose DP, Connolly JM, Coleman M. Effect of omega-3 fatty acids on the progression of metastases after the surgical excision of human breast cancer cell solid tumors growing in nude mice. Clin Cancer Res 1996;2:1751-56.

11. Jenski LJ, Zerouga M, Stillwell W. Omega-3 fatty acid-containing liposomes in cancer therapy. Proc Soc Exp Biol Med 1995;210:227-33.

12. Norrish AE, Skeaff CM, Arribas GL, Sharpe SJ, Jackson RT. Prostate cancer risk and consumption of fish oils: a dietary biomarker-based case-control study. British Journal of Cancer 1999;81:1238-42.

13. Calviello G, Palozza P, Maggiano N, Franceschelli P, Di Nicuolo F, Marcocci ME, Bartoli GM. Effects of eicosapentaenoic and docosahexaenoic acids dietary supplementation on cell proliferation and apoptosis in rat colonic mucosa. Lipids 1999;Suppl 34:S111.

14. Latham P, Lund EK, Johnson IT. Dietary n-3 PUFA increases the apoptotic response to 1,2- dimethylhydrazine, reduces mitosis and suppresses the induction of carcinogenesis in the rat colon. Carcinogenesis 1999;20:645-50.

15. Calviello G, Palozza P, Maggiano N, Piccioni E, Franceschelli P, Frattucci A, Di Nicuolo F, Bartoli GM. Cell proliferation, differentiation, and apoptosis are modified by n-3 polyunsaturated fatty acids in normal colonic mucosa. Lipids 1999;34:599-604.

16. Collett ED. Docosahexaenoic acid inhibits oncogenic Ras activation and signal transduction in colonocytes. FASEB J 2000;14:A169.

17. Hong MY, Chapkin RS, Davidson LA, Turner ND, Morris JS, Wang N, Carroll RJ, Lupton JR. Fish oil enhances targeted apoptosis during colon tumor initiation by downregulating ECL-3. FASEB J 2000;14:A169.

18. Calviello G, Palozza P, Piccioni E, Maggiano N, Frattucci A, Franceschelli P, Bartoli GM. Dietary supplementation with eicosapentaenoic and docosahexaenoic acid inhibits growth of Morris hepatocarcinoma 3924A in rats: effects on proliferation and apoptosis. Int J Cancer 1998;75:699-705.

19. Hatala MA, Rayburn J, Rose DP. Comparison of linoleic acid and eicosapentaenoic acid incorporation into human breast cancer cells. Lipids 1994;29:831-37.

20. Rose DP, Connolly JM. Effects of fatty acids and inhibitors of eicosanoid synthesis on the growth of a human breast cancer cell line in culture. Cancer Res 1990;50:7139-44.

21. Madhavi N, Das UN. Effect of n-6 and n-3 fatty acids on the survival of vincristine sensitive and resistant human cervical carcinoma cells in vitro. Cancer Lett 1994;84:31-41.

22. Siddiqui RA, Jenski LJ, Neff K, Harvey K, Kovacs R, Stillwell W. Docosahexanoic acid induces apoptosis in Jurkat cells by a protein phosphatase-mediated process. Biochim Biophys Acta 2001;1499:265-75.

23. Siddiqui R, Jenski L, Harvey K, Wiesehan JD, Stillwell W, Zaloga GP. Cell cycle arrest in jurkat leukemic cells: a possible role for docosahexaenoic acid. Biochem J 2003;371:621-29.

24. Siddiqui RA, Jenski LJ, Wiesehan JD, Hunter MV, Kovacs RJ, Stillwell W. Prevention of docosahexaenoic acid-induced cytotoxicity by phosphatidic acid in Jurkat leukemic cells: the role of protein phosphatase-1. Biochim Biophys Acta 2001;1541:188-200.

25. Tonnetti L, Veri MC, Bonvini E, D'Adamio L. A role for neutral sphingomyelinase-mediated ceramide production in T cell receptor-induced apoptosis and mitogen-activated protein kinase- mediated signal transduction. J Exp Med 1999;189:1581-9.

26. Jayadev S, Liu B, Bielawska AE, Lee JY, Nazaire F, Pushkareva M, Obeid LM, Hannun YA. Role for ceramide in cell cycle arrest. Journal of Biological Chemistry 1995;270:2047-52.

27. Spence MW. Sphingomyelinases. Adv Lipid Res 1993;26:3-23.

28. Levade T, Jaffrezou JP. Signalling sphingomyelinases: which, where, how and why? Biochim Biophys Acta 1999;1438:1-17.

29. Okazaki T, Kondo T, Kitano T, Tashima M. Diversity and complexity of ceramide signalling in apoptosis. Cell Signal 1998;10:685-92.

30. Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Fuks Z, et al. Requirement for ceramide-initiated SAPK/JNK signalling in stress- induced apoptosis. Nature 1996;380:75-9.

31. Sandhoff K, Klein A. Intracellular trafficking of glycosphingolipids: role of sphingolipid activator proteins in the topology of endocytosis and lysosomal digestion. FEBS Lett 1994;346:103-7.

32. Hostetler KY, Yazaki PJ. The subcellular localization of neutral sphingomyelinase in rat liver. J Lipid Res 1979;20:456-63.

33. Marchesini N, Osta W, Bielawski J, Luberto C, Obeid LM, Hannun YA. Role for mammalian neutral sphingomyelinase 2 in confluence-induced growth arrest of MCF7 cells. J Biol Chem 2004:279:25101-11.

34. Veldman RJ, Maestre N, Aduib OM, Medin JA, Salvayre R, Levade T. A neutral sphingomyelinase resides in sphingolipid-enriched microdomains and is inhibited by the caveolin-scaffolding domain: potential implications in tumour necrosis factor signalling. Biochem J 2001;355:859-68.

35. Shaikh SA, Dumaual AC, Castillo A, LoCascio D, Siddiqui RA, Stillwell W, Wassall SR. Oleic and Docosahexaenoic Acid Differentially Phase Separate form Lipid Raft Molecules: A Comparative NMR, DSC, AFM, and Detergent Extraction Study. Boiphysical Journal 2004;87:1752-66.

36. Lopez-Marure R, Ventura JL, Sanchez L, Montano LF, Zentella A. Ceramide mimics tumour necrosis factor-alpha in the induction of cell cycle arrest in endothelial cells. Induction of the tumour suppressor p53 with decrease in retinoblastoma/protein levels. European Journal of Biochemistry 2000;267:4325-33.

37. Lee JY, Leonhardt LG, Obeid LM. Cell-cycle-dependent changes in ceramide levels preceding retinoblastoma protein dephosphorylation in G2/M. Biochemical Journal 1998;334:457-61.

38. Lee JY, Bielawska AE, Obeid LM. Regulation of cyclin-dependent kinase 2 activity by ceramide. Exp Cell Res 2000;261:303-11.

39. Hellin AC, Bentires-Alj M, Verlaet M, Benoit V, Gielen J, Bours V, Merville MP. Roles of nuclear factor-kappaB, p53, and p21/WAF1 in daunomycin-induced cell cycle arrest and apoptosis. Journal of Pharmacology & Experimental Therapeutics 2000;295:870-8.

40. Guaitani A, Recchia M, Carli M, Rocchetti M, Bartosek I, Garattini S. Walker carcinoma 256: a model for studies on tumor-induced anorexia and cachexia. Oncology 1982;39:173-8.

41. Vielhaber G BL, Lindner B, Pfeiffer S, Wepf R, Hintze U, Wittern KP, Brade H,. Mouse anti-ceramide antiserum: a specific tool for the detection of endogenous ceramide. Glycobiology 2001;11:451-7.

42. 2004 CFF. Cancer Facts & Figures 2004. American Cancer Society, Inc., Atlanta, GA 2004.

43. Hannun YA, Bell RM. Functions of sphingolipids and sphingolipid breakdown products in cellular regulation.[see comment]. [Review] [110 refs]. Science 1989;243:500-7.

44. Kolesnick RN. Sphingomyelin and derivatives as cellular signals. Prog Lipid Res 1991:30:1-38.

45. Liu B, Andrieu-Abadie N, Levade T, Zhang P, Obeid LM, Hannun YA. Glutathione regulation of neutral sphingomyelinase in tumor necrosis factor-alpha-induced cell death. J Biol Chem 1998:273:11313-20.

46. Tepper CG, Jayadev S, Liu B, Bielawska A, Wolff R, Yonehara S, Hannun YA, Seldin MF. Role for ceramide as an endogenous mediator of Fas-induced cytotoxicity. Proc Natl Acad Sci U S A 1995;92:8443-7.

47. Hannun YA. The sphingomyelin cycle and the second messenger function of ceramide. J Biol Chem 1994;269:3125-8.

48. Hannun YA. Functions of ceramide in coordinating cellular responses to stress. Science 1996;274:1855-9.

49. van Blitterswijk WJ, van der Luit AH, Veldman RJ, Verheij M, Borst J. Ceramide: second messenger or modulator of membrane structure and dynamics? Biochem J 2003:369(Pt 2):199-211.

50. Prinetti A, Chigorno V, Prioni S, Loberto N, Marano N, Tettamanti G, Sonnino S. Changes in the lipid turnover, composition, and organization, as sphingolipid-enriched membrane domains, in rat cerebellar granule cells developing in vitro. J Biol Chem 2001:276:21136-45.

51. Grassme H, Jekle A, Riehle A, Schwarz H, Berger J, Sandhoff K, Kolesnick R, Gulbins E. CD95 signaling via ceramide-rich membrane rafts. J Biol Chem 2001;276:20589-96.

52. Adam-Klages S, Adam D, Wiegmann K, Struve S, Kolanus W, Schneider-Mergener J, Kronke M. FAN, a novel WD-repeat protein, couples the p55 TNF-receptor to neutral sphingomyelinase. Cell 1996;86:937-47.

53. Tepper AD, Ruurs P, Wiedmer T, Sims PJ, Borst J, van Blitterswijk WJ. Sphingomyelin hydrolysis to ceramide during the execution phase of apoptosis results from phospholipid scrambling and alters cell-surface morphology.[see comment]. J Cell Biol 2000:150:155-64.

Table 1: Experimental Diets

	n-6 Diet	n-3 Diet
Carbohydrates (% calories)	58.8	58.8
Starch (g/100g)	25.0	25.0
Maltodextrins (g/100g)	5.0	5.0
Sucrose (g/100g)	30.0	30.0
Cellulose (g/100g)	5.0	5.0
Protein (% calories)	19.6	19.6
Casein (g/100g)	20.2	20.2
DL-methionine (g/100g)	0.3	0.3
Lipid (% calories)	21.7	21.7
Corn oil (g/100g)	10.0	1.0*
Fish oil (g/100g)	0	9.0*
n-6/n-3 ratio	72.5	0.11*
Mineral mix (g/100g)	3.45	3.45
Vitamin mix (g/100g)	1.0	1.0
Vitamin E (g/100g)	0.03	0.03
DHT (g/100g)	0.02	0.02

*Differs between diets

Table 2: Fatty acid composition of dietary oils

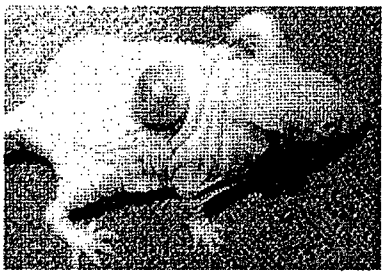
Fatty acids	Corn oil (%)	Fish oil (%)
14:0		8.0
16:0	12.0	17.0
16:1		14.0
18:0	7.0	4.0
18:1n-9	22.0	26.0
18:2n-6	58.2	2.0
18:3n-3	0.8	3.0
18:4n-3		3.0
20:4n-6		1.0
20:5n-3		12.0
22:6n-3		10.0
Saturated fatty acids	19.0	29.0
Monounsaturated fatty acids	22.0	40.0
Total n-6 lipids	58.2	3.0
Total n-3 lipids	0.8	28.0
n-6/n-3 ratio	72	0.11

Table 3: Effect of corn oil and fish oil diets on breast cancer growth in nude mice

Diet	Tumor volume (mm^3)	Surface area (mm^2)	Tumor Weight (mg)
Corn Oil	307.7±7.0	76.9±3.2	180±10
Balanced Corn/Fish Oil fed	114.5±17.3*	49.8±7.4*	130±20*
Fish Oil	75.5±6.9*	38.9±3.3*	90±10*

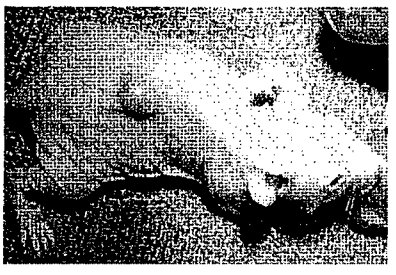
Results are the mean ± SEM for three experiments. Results were analyzed by Student's t-test and one-way ANOVA. Significant differences compared to the Corn Oil group are reported (*$P<0.05$).

a



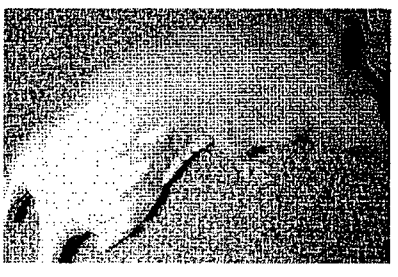
Corn Oil

b



Balanced Corn/Fish Oil

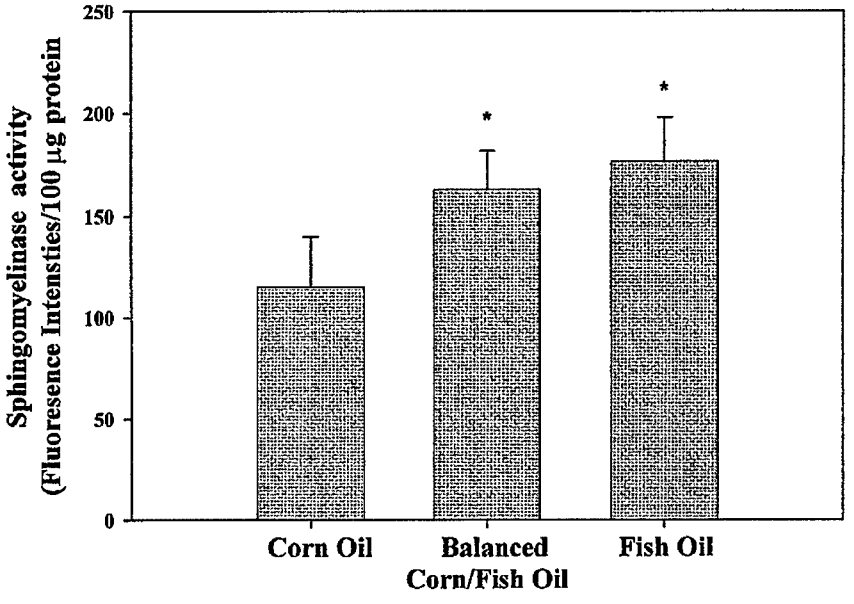
c



Fish Oil

Figure 1

a


Sphingomyelinase activity
(Fluoresence Intensties/100 µg protein
250
200
150
100
50
0
*
*
Corn Oil
Balanced
Corn/Fish Oil
Fish Oil

b

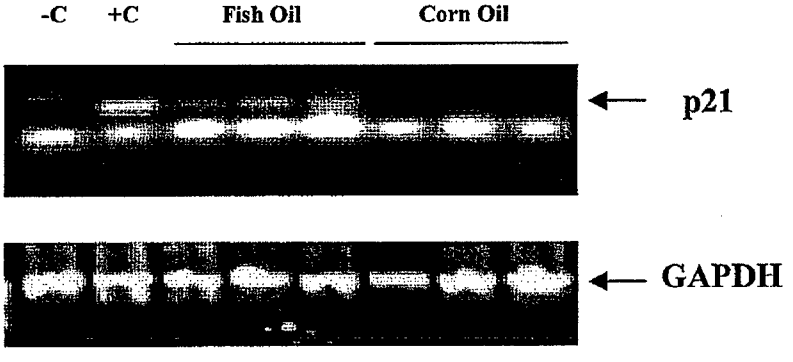

-C
+C
Fish Oil
Corn Oil
p21
GAPDH

Figure 2

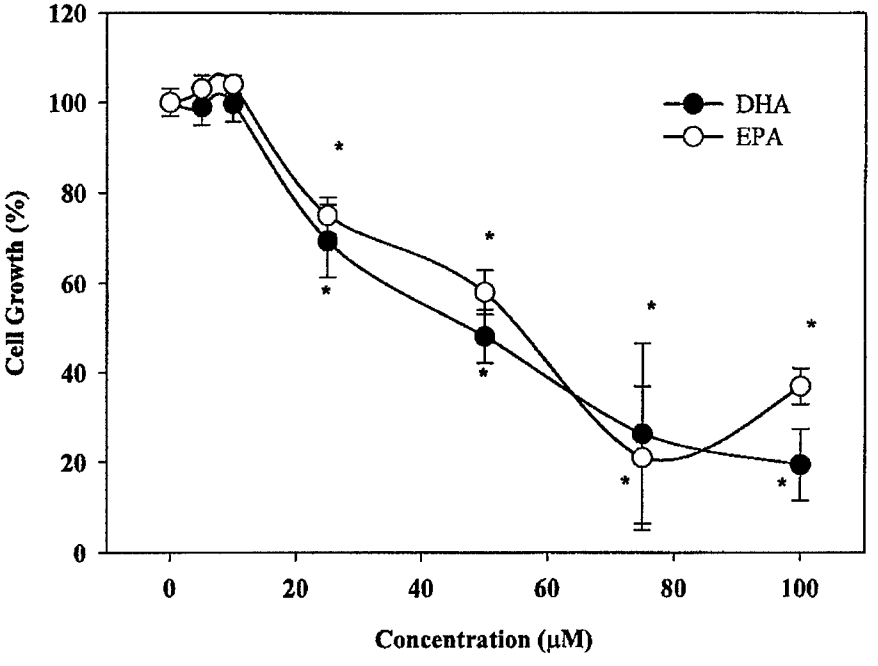

120
100
80
60
40
20
0
Cell Growth (%)
0
20
40
60
80
100
Concentration (μM)
DHA
EPA

Figure 3

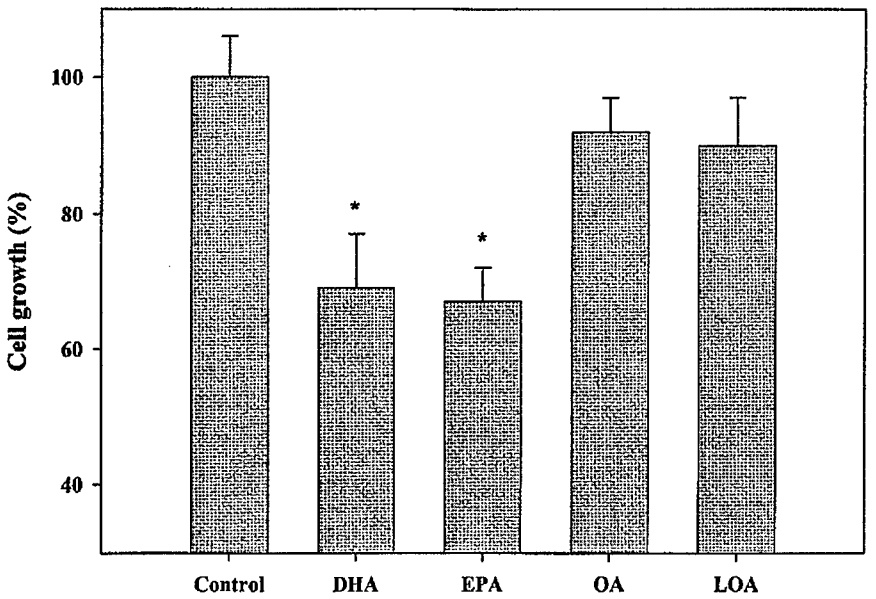
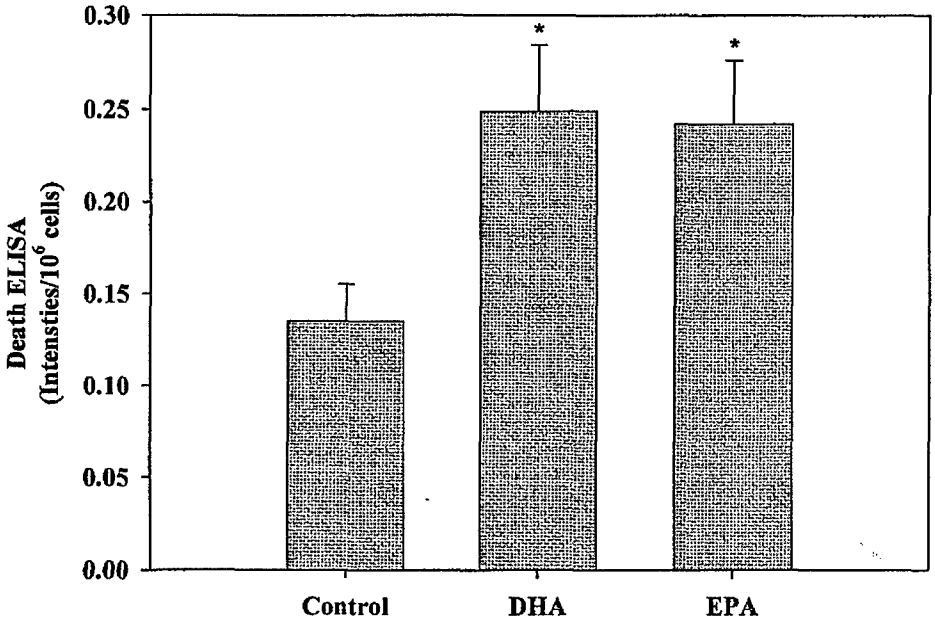


Figure 4

a


0.30
0.25
0.20
0.15
0.10
0.05
0.00
Death ELISA
(Intensties/10^6 cells)
*
*
Control
DHA
EPA

b

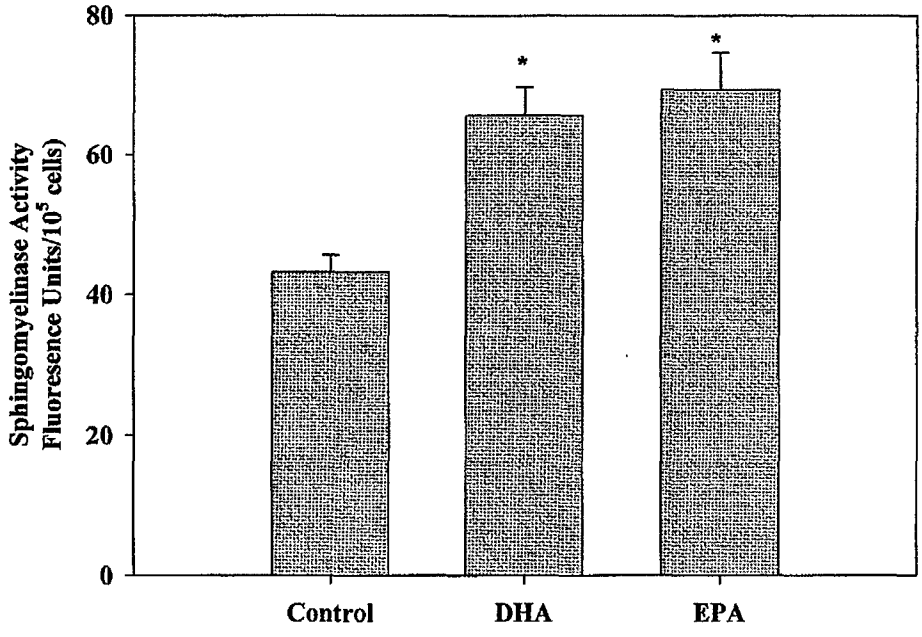
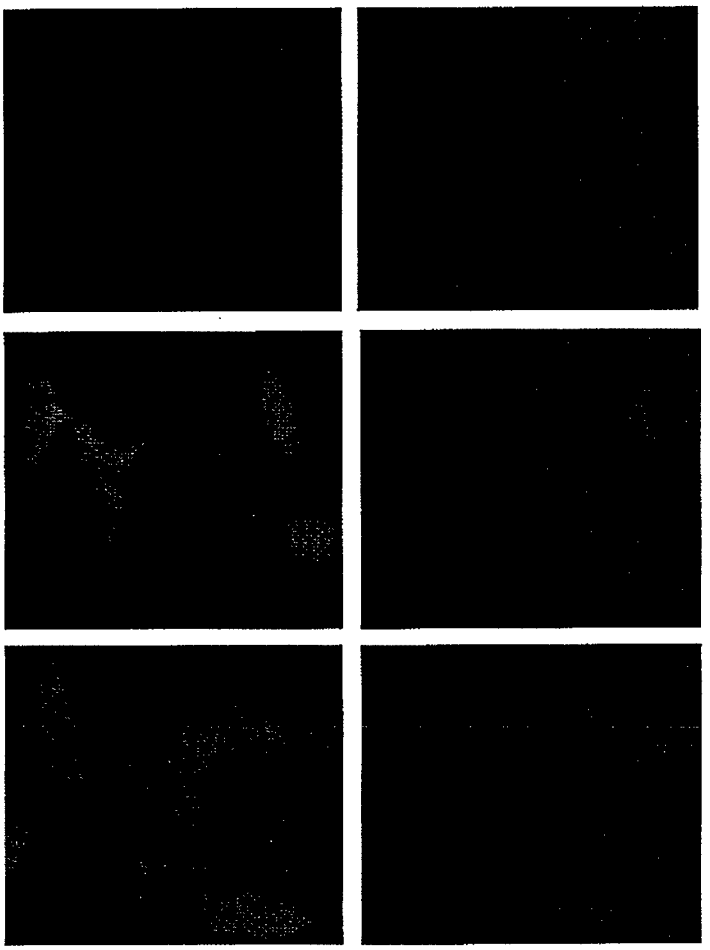
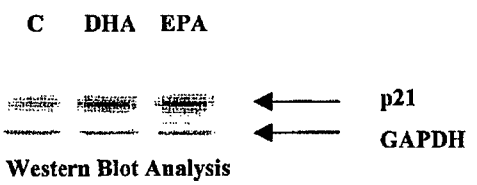

80
60
40
20
0
Sphingomyelinase Activity
Fluoresence Units/10^5 cells)
*
*
Control
DHA
EPA

Figure 5

a


Ceramide Staining
DAPI Staining
Control
DHA
(25 μM)
EPA
(25 μM)

b


C
DHA
EPA
p21
GAPDH
Western Blot Analysis

c

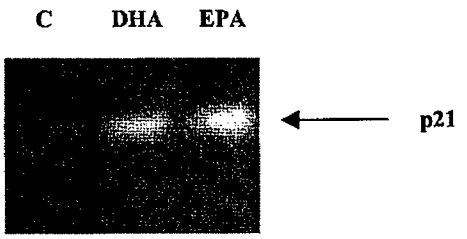
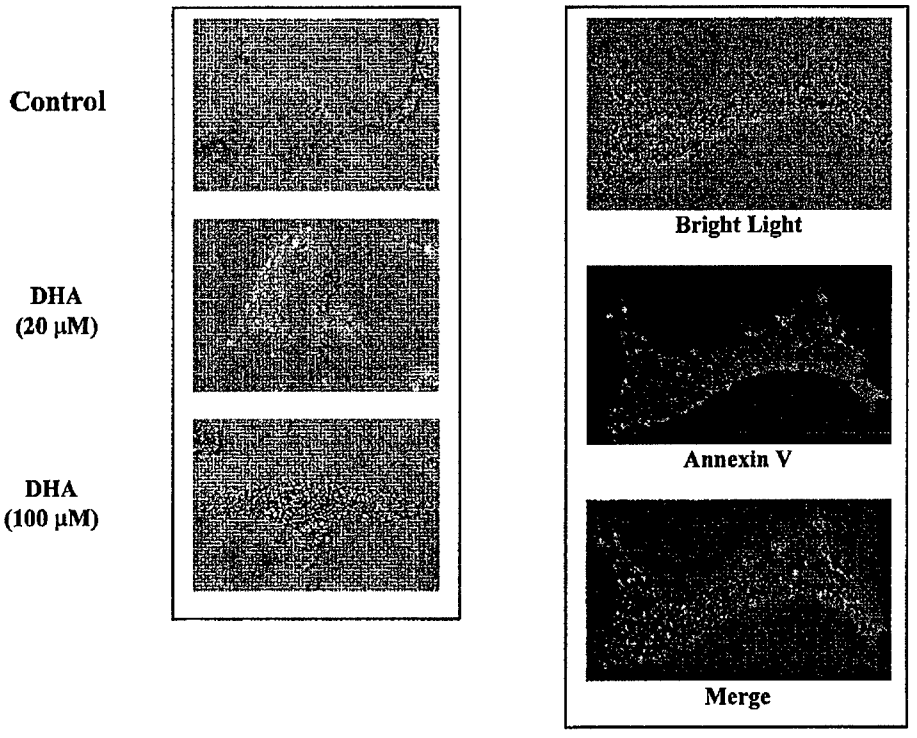

C
DHA
EPA
p21
a
b
RT-PCR

Figure 6


Control
DHA
(20 μM)
DHA
(100 μM)
Bright Light
Annexin V
Merge

c

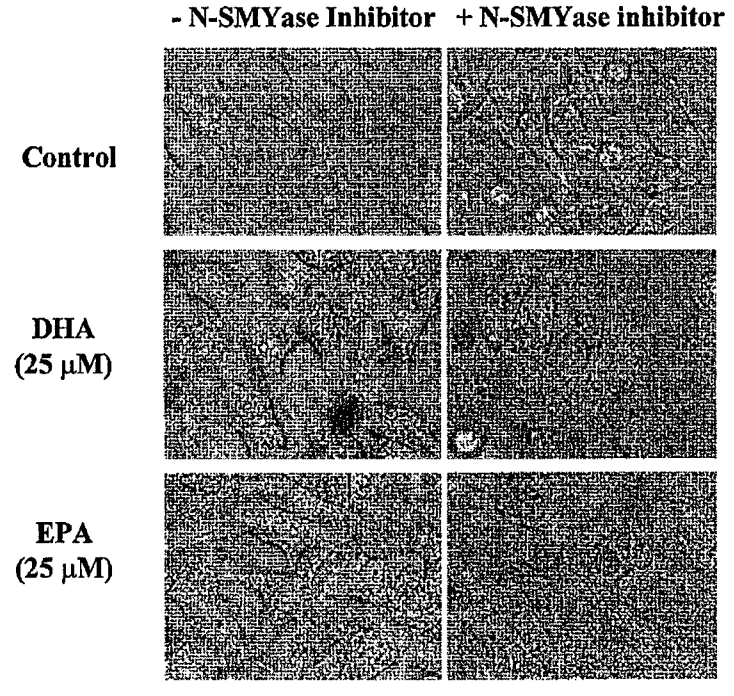
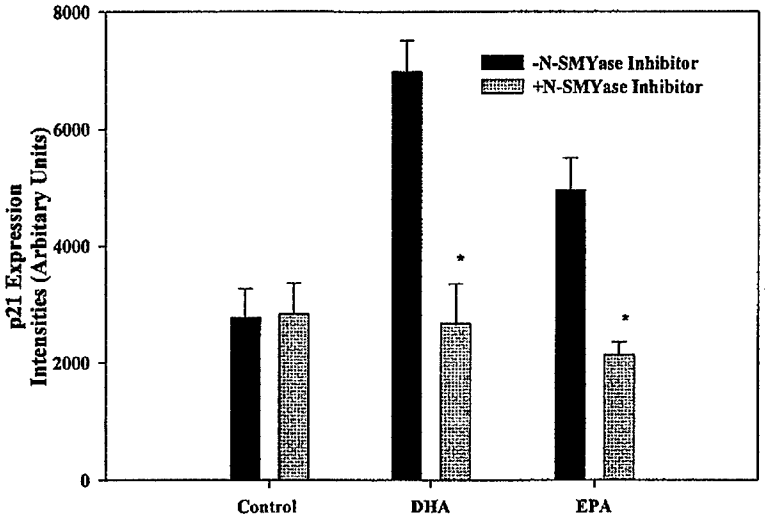

- N-SMYase Inhibitor
+ N-SMYase inhibitor
Control
DHA
(25 μM)
EPA
(25 μM)

Figure 7

a


8000
6000
4000
2000
0
p21 Expression Intensities (Arbitary Units)
-N-SMYase Inhibitor
+N-SMYase Inhibitor
*
*
Control
DHA
EPA

b

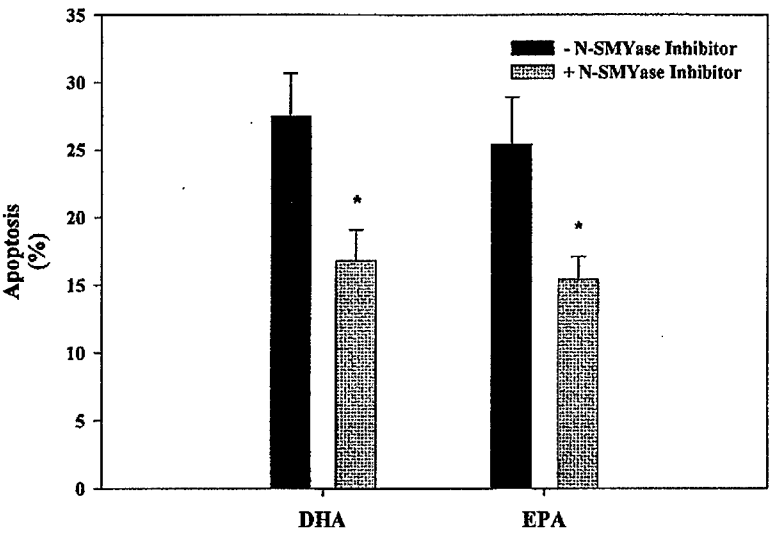

35
30
25
20
15
10
5
0
Apoptosis (%)
- N-SMYase Inhibitor
+ N-SMYase Inhibitor
*
*
DHA
EPA

Figure 8

SU1498, AN INHIBITOR OF VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR 2 AND ERK1/2, PREVENTS DEPHOSPHORYLATION OF PHOSPHO-ERK AND PROMOTES APOPTOSIS*

George Boguslawski[‡], Patrick W. McGlynn[‡], Jennifer Griffith[‡], Kevin A. Harvey[‡] and A. Thomas Kovala[‡¶†]

[‡]Methodist Research Institute, Clarian Health Partners and [¶]Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana 46202

Running title: Inhibition of ERK1/2 by SU1498

***Supported** by a grant from Methodist Heart Institute, Cardiovascular Trust Fund, to A. T. Kovala. Jennifer Griffith's contribution to the project was supported by a Breast Cancer Research Training Program grant (#BC020180) from the Department of the Army Medical Research and Materiel Command.

[†] **Corresponding author**. Present address: Northern Ontario School of Medicine, Laurentian University, Willet Green Miller Centre, Sudbury, ON, Canada P3E 2C6

Telephone: 705-662-7237

Fax: 705-675-4858

mailto: Tom.Kovala@NorMed.ca

SUMMARY

Most cells, when stimulated with sphingosine 1-phosphate, transiently accumulate phosphorylated ERK. We previously observed that in endothelial cells treated with sphingosine 1-phosphate, SU1498, a well-known powerful inhibitor of VEGF receptor 2, while not interfering with phosphorylation of ERK1/2, prevented the subsequent decrease in the level of phosphorylated ERK species. As a result, there was a net accumulation of P-ERK1/2 in these cells. At the same time, the kinase activity of ERK1/2 was inhibited by SU1498. On the basis of these results we proposed a model of SU1498 action according to which the inhibitor associates with phosphorylated ERK and renders it inaccessible to an ERK-specific phosphatase. We now show that this model is correct and that SU1498 prevents phosphorylated ERK2 from being dephosphorylated by the phosphatase MKP-1. The inhibitor does not affect the phosphatase itself. We show that although SU1498 does not promote phospho-ERK accumulation in smooth muscle cells or breast cancer cells, the kinase activity of phospho-ERKs in whole lysates or immunoprecipitates from these cells is strongly inhibited by the compound. We further demonstrate that SU1498 exerts an apoptotic effect on breast cancer cells, a finding consistent with the essentiality of ERKs in cellular growth and proliferation.

INTRODUCTION

Proliferative and apoptotic events accompanying the cell life cycle are the areas of intense study. It is the balance between these events that determines the cell's fate. Broadly speaking, proliferation is stimulated by so-called growth factors, a large group of substances that includes proteinaceous molecules such as EGF[1], VEGF, PDGF, and

others. Another group of growth factors includes biologically active lipids such as sphingosine 1-phosphate (S1P) and lysophosphatidic acid. All these factors interact with cells through specific protein tyrosine kinase or G protein-coupled receptors (1,2), one of the most immediate results of the interaction being the activation of MAPK signaling pathways of which ERKs are a major component (3). The ERKs are activated by dual (Thr–Tyr) phosphorylation, and can be de-activated through the removal of phosphate groups by one of a number of dual specificity phosphatases such as MKP-1 and MKP-3 (4-7) or the tyrosine phosphatases HePTP, PTP-SL or STEP (8,9).

Apoptotic events are generally initiated by a variety of stresses and/or DNA damage. The major routes of programmed cell death are caspase-dependent (10-12), although there is evidence for caspase-independent pathways (13-15). The caspase-dependent events can be either extrinsic (responding to death receptor-ligand interactions) or intrinsic (responding to internal damage and involving mitochondria and formation of apoptosome) (16). Interference with proliferative pathways can have an effect of inducing apoptotic responses (17,18), and agents that block MAPK signaling are known to act pro-apoptotically (19-21). Since our experiments demonstrated that SU1498 interferes with the functioning of ERKs, we examined the apoptotic activity of the inhibitor. In the work described here we present evidence that SU1498, originally described as a specific VEGF receptor 2 inhibitor (22), inactivates ERK1/2, prevents their dephosphorylation by the phosphatase MKP-1 and promotes apoptosis in breast cancer cells.

EXPERIMENTAL PROCEDURES

Materials, chemicals and antibodies - Media components were from Gibco Invitrogen (Invitrogen Corp., Grand Island, NY) and from Cambrex Bio Science (Walkersville, MD). Culture flasks and plates were purchased from Fisher Scientific (Chicago, IL). Precast polyacrylamide gels, electrophoresis buffers and nitrocellulose membranes were purchased from Invitrogen Corp. (Carlsbad, CA), and Western Blocking Reagent was from Roche Molecular Biochemicals (Indianapolis, IN). Sphingosine 1-phosphate was from Sigma Chemical Co. (St Louis, MO) and the various inhibitors used in this study, including SU1498, were obtained from Calbiochem (La Jolla, CA). Protein growth factor EGF was from R&D Systems (Minneapolis, MN). Rabbit polyclonal anti-ERK1/2 and anti-phospho-ERK1/2 antibodies were from Cell Signaling Technology (Beverly, MA), as was the p44/p42 MAP kinase assay kit that included activated murine ERK2, the Elk1 fusion protein substrate (41 kDa) and anti-phospho-Elk1 antibody. Purified activated murine ERK2 was also separately purchased from New England Biolabs (Beverly, MA). Active p38α/SAPK2a kinase (hereinafter referred to as p38 kinase) and MKP-1/CL-100 phosphatase were acquired from Upstate (Charlottesville, VA).

Cell cultures - Human umbilical vein endothelial cells (HUVEC) were obtained from VEC Technologies, Inc. (Rensselaer, NY) and grown up to no more than passage 10 in RPMI 1640- or DMEM-based medium (Gibco Invitrogen) containing endothelial cell growth supplement (30 μg/ml; Upstate), heparin sulfate (2 units/ml), 25 mM glucose, 20 mM L-glutamine, antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml;

amphotericin B, 0.25 μg/ml) and 20% fetal bovine serum (FBS). When necessary, cells were starved in the same medium containing 0.5% FBS. Human aortic smooth muscle cells (HASMC) were obtained from Cambrex Bio Science and were grown up to passage 10 in SMBM medium containing 10% FBS and SingleQuot kit supplement, except that gentamycin and amphotericin were replaced by the antibiotics listed above. Starvation was in the same medium containing 0.1% serum.

Cell lines MCF-7 and MDA-MB-231 derived from the human mammary gland were obtained from the American Type Culture Collection, Manassas, VA. Two additional cell lines, MCF-10A and MDA-MB-468, were kindly provided by Linda Malkas (Indiana University Medical Center, Cancer Research Institute, Indianapolis, IN). MCF-10A is a non-tumorigenic epithelial cell line; MCF-7 is an adenocarcinoma line that is mildly invasive; MDA-MB-231 and MDA-MB-468 are highly invasive adenocarcinoma cells. The MDA-MB-231 cells, MDA-MB-468 cells and MCF-7 cells were grown in DMEM (high glucose) with L-glutamine, 110 mg/l sodium pyruvate and pyridoxine hydrochloride, and containing antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml; amphotericin B, 0.25 μg/ml) and 10% FBS. For starvation, the cells were incubated in the same medium containing 0.1% FBS, except for the experiment shown in Fig. 6, where no FBS was present. The MCF-10A cells were maintained in a medium consisting of 1:1 mixture of DMEM/F12, supplemented with 5% equine serum, insulin (10 μg/ml), EGF (20 ng/ml), cholera toxin (100 ng/ml), hydrocortisone (0.5 μg/ml), and the antibiotics listed above. Starvation medium was the

same except that insulin and EGF were omitted and the equine serum was replaced by 0.1% FBS.

Preparation of cell lysates, SDS-PAGE and Western blotting - Cells grown in plates or flasks were serum-starved, as indicated, and rinsed with PBS. Following treatments described in Figure legends, the cells were lysed with the lysis buffer (50 mM Tris-HCl, pH 7.2-7.4; 1% Triton X-100; 0.5 M NaCl; 0.125% sodium deoxycholate; 0.1% SDS; 10 mM $MgCl_2$) containing 2 mM NaF, 0.2 mM sodium pervanadate, aprotinin (20 μg/ml), leupeptin (20 μg/ml) and 1 mM PMSF. Lysates were mixed with one-fourth the volume of the 4X electrophoresis sample buffer (Invitrogen) containing 1% mercaptoethanol, heated 5 min at 95 °C, and stored at –80° C until use. Samples of the lysates were fractionated by SDS-PAGE, proteins transferred electrophoretically to nitrocellulose membranes, blocked with 10% Western Blocking Reagent in 0.1% Tween20 in Tris-buffered saline (TBST) and probed with appropriate antibodies. The signal was detected using goat anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugates and the ECL-Plus chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ).

To prepare whole cell lysates suitable for ERK activity tests, the SDS- and deoxycholate-free lysis buffer provided in the ERK assay kit was used instead of the buffer described above. The cells were lysed and the lysates were adjusted to the same protein concentration (approximately 20 μg of protein per assay). Each reaction mixture also contained 1X kinase buffer, 250 μM ATP and 400 ng of Elk1 peptide in the total

volume of 40 µl. The activity was measured as the ability to phosphorylate Elk1 protein that was detected with anti-phospho-Elk1-specific antibody (see below).

ERK and p38 kinase activity assays (purified enzymes) - A kit from Cell Signaling was used for this purpose. This kit included the kinase buffer, ATP, and the kinase protein substrate, Elk1, whose phosphorylation was detected with anti-phospho-Elk1 antibody. The active ERK2 or p38 kinase was diluted to 1 ng/µl or 5 ng/µl, respectively, with the kinase buffer without ATP. Separately, the Elk1 protein substrate was diluted to 20 ng/µl with the kinase buffer containing 50 µM ATP. The ERK2 or p38 kinase solution was pipetted into tubes (2 µl per tube) and mixed with an appropriate amount of SU1498 or SB203580-iodo (in kinase buffer without ATP). The blank tube received buffer only (no enzyme). The volume was adjusted to 10 µl with the same buffer and the mixtures were incubated for 10 min at 25° C. This was followed by the addition of 40 µl of the Elk1-ATP-buffer solution, and the incubations continued for 30 min at 30° C. The reactions were stopped with 20 µl of 4X sample buffer mix and heating at 95° C for 10 minutes. Samples (10 µl) were fractionated by SDS-PAGE and phosphorylated Elk1 was detected by immunoblotting with anti-phospho-Elk1 antibody.

Immunoprecipitation assay - The assay was performed essentially as instructed by the manufacturer. Appropriate cells were grown in T150 culture flasks (2 x 10^6 cells per flask) and starved overnight. The medium was siphoned off, and the cells were washed with PBS and overlaid with 4 ml of DMEM. SU1498 was added to selected flasks for 15 min, followed by S1P (1 µM). Where other inhibitors were used, they were added to

flasks 15 min before SU1498. A control flask was left untreated. After a 10 min incubation with S1P, the cells were lysed with 0.8 ml of 1X lysis buffer provided with the kit, and the clear supernatants were harvested by centrifugation for 10 min at 15,000 x g at 4° C. A portion of each supernatant was mixed with one-fourth the volume of 4X sample buffer mix, heated at 95° C and used to detect phospho-ERK1/2 and total ERK1/2 by Western blotting. The remaining supernatants (in a volume supplying approximately 200-300 μg of protein each) received a 10-15 μl aliquot of immobilized anti-P-ERK antibody, and the suspensions were incubated with gentle agitation overnight at 4° C. The precipitates were collected by centrifugation and the pellets were suspended in 40 μl of the kinase buffer containing 200 μM ATP and 400 ng of Elk1 protein. The mixtures were incubated 30 min at 30° C and the reactions were stopped with 20 μl of 4X sample buffer and heated at 95° C. Samples (10-15 μl) were fractionated by SDS-PAGE and phosphorylated Elk1 was detected by immunoblotting with anti-phospho-Elk1 antibody.

MKP-1 phosphatase assay - The enzyme activity was measured as the ability to dephosphorylate P-ERK2. The phosphatase (10 units per assay point) and its substrate (phosphorylated ERK2, 5 ng per assay point) were incubated in phosphatase buffer (100 mM Tris-HCl, pH 8.2; 40 mM NaCl; 1 mM dithiothreitol; 20 % glycerol) in scaled-up reactions at 30° C, and the reactions were terminated at indicated times by removing 10 μl aliquots into 3 μl of 4X sample buffer followed by heating at 95° C. Where indicated, SU1498 (20 μM) was included; either the phosphatase or the P-ERK2 substrate were preincubated with the inhibitor for 10 min at 25° C before the start of the reaction. The enzymes without the inhibitor were also preincubated for 10 min at 25° C. The remaining

phosphorylated ERK2 was detected by Western blotting (6 µl samples), using a phospho-ERK-specific antibody.

Apoptosis assay - Cells were grown in T150 flasks to 80-90% confluence and rinsed twice with PBS. The cells were suspended by trypsinization, washed by centrifugation (5 min, 2,000 x *g*), and inoculated into 6-well plates (5 x 10^5 cells/well) containing the appropriate growth medium. The plates were incubated at 37° C in the atmosphere of 5% CO_2. After 24 hrs, the medium in wells was replaced with either starvation medium (0.1% FBS) or normal medium. Where indicated, 10 µM SU1498 was also included. Following the additional 24 hr incubation, the media were aspirated and collected in polystyrene tubes. The cells from each well were then suspended by trypsinization and combined with the appropriate saved medium. After a brief centrifugation, the cell pellets were washed with PBS and were labeled using the Annexin-V-FLUOS Staining Kit (Roche). The manufacturer's instructions were followed throughout. Cells were suspended in 100 µl labeling solution, and the labeled samples were incubated for 15 min in the dark, after which 200 µl of incubation buffer were added to each sample to bring the volume up for analysis. Cytometric analysis was performed using a FACSCalibur flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA) equipped with an air-cooled argon laser emitting at 488 nm. Quantitative analysis was performed on 10,000 events per sample, using CELLQuest Version 3.1f software (Becton-Dickinson).

Mitochondrial membrane potential ($\Delta\psi_m$) detection – Cells were plated in Lab-Tek chamber slides (Nalge-Nunc, Napperville, IL) at a density of 10^5 cells per chamber and incubated overnight in appropriate media. The cells were then starved overnight in the absence or presence of 10 μM SU1498 and the loss of mitochondrial membrane potential, $\Delta\psi_m$, was detected using Apo Logix JC-1 kit (Cell Technology Inc., Minneapolis, MN) according to the manufacturer's instructions. The JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) yields red-fluorescing (590 nm) aggregates in normal mitochondria but remains in a green-fluorescing (530 nm) monomeric form in cells in which $\Delta\psi_m$ has collapsed. The images were obtained with the help of a Leica DMR fluorescence microscope and the Spot Advance software (Diagnostic Instruments, Sterling Heights, MI).

RESULTS

SU1498 does not inhibit p38 kinase - In the earlier communication, we demonstrated that SU1498 enhanced accumulation of phosphorylated ERK1/2 in endothelial cells *in vivo* and inhibited the kinase activity *in vitro* (23). We wanted to ascertain that SU1498 is not a promiscuous inhibitor of MAP kinases and compared its action with that of a compound SB203580-iodo, an inhibitor of stress-activated protein kinase p38 (24). As shown in Fig. 1, while SU1498 inhibited ERK2 activity, it had no effect on the activity of p38 kinase. Conversely, p38 kinase activity was eliminated by SB203580-iodo, but was unaffected by SU1498 even at 16 μM concentration.

SU1498 protects P-ERK2 from dephosphorylation by MKP-1 - Having established the selectivity of the SU1498 effect, we tested the model of its action that was proposed earlier for endothelial cells (23). Generally, MAP kinases are regulated by phosphorylation-dephosphorylation equilibrium (4,25), and there are MAP kinase-specific phosphatases that control MAPK phosphorylation state. Interestingly, the expression of some of these phosphatases is the consequence of ERK activation (25,26). We hypothesized that the inhibitor binds to the phosphorylated ERK and prevents its dephosphorylation by hindering the access to it by a P-ERK-specific phosphatase. Alternatively, we considered that the inhibitor blocks phosphatase activity directly, the net result in either case being an accumulation of P-ERK in cells exposed to SU1498.

We used a dual specificity phosphatase MKP-1/CL100, originally described by Zheng *et al.* (6), to test the above model. First, we ascertained that in the ERK2 activity assay we employed, the phosphatase does not promiscuously dephosphorylate the kinase reaction product, phosphorylated Elk-1 peptide. Fig. 2A demonstrates that, as expected, adding MKP-1 phosphatase to the reaction mix at time 0 abolished Elk-1 phosphorylation. However, when the phosphatase was added after the reaction had run its course (30 min), the P-Elk peptide remained phosphorylated even after the additional 30 min of incubation. Thus, the phosphatase does indeed abolish ERK2 activity, presumably by dephosphorylating the essential Thr-Tyr residues of ERK2 protein.

Fig. 2B shows that MKP-1 dephosphorylates P-ERK2 in a time-dependent manner. The top panel in Fig. 2B compares the activity of untreated MKP-1 with that of

the SU1498-treated enzyme. It is clear that SU1498 does not interfere with the ability of the phosphatase to dephosphorylate the P-ERK2 substrate. In contrast (Fig. 2B, bottom panel), preincubation of P-ERK2 with SU1498 rendered it resistant to dephosphorylation. Note that in this part of the experiment, a portion of the input P-ERK2 was acted upon by the phosphatase within the first minute of incubation; this fraction was not protected by SU1498 against MPK-1 action because the amount of P-ERK in the reaction was too large for SU1498 to saturate. When SU1498 concentration was raised to 40 μM, phospho-ERK2 was well protected (Fig. 2C). These results confirm the validity of the proposed model.

Different cell lines respond differently to treatment with SU1498 - We observed previously that – unlike the endothelial cells (HUVECs) – human aortic smooth muscle cells (HASMCs) did not accumulate P-ERK1/2 in response to treatment with SU1498 (23). This lack of response could be caused by a difference in the manner in which ERKs are regulated in HASMCs or by the qualitative and/or quantitative distinctiveness of ERKs in these cells. To address this issue, we tested whole cell lysates from HUVECs and HASMCs for the accumulation of P-ERK1/2 as well as for the enzymatic activity of ERK (as reflected in the ability to phosphorylate Elk). Fig. 3 shows that, as expected, treatment of S1P-stimulated HUVECs with SU1498 resulted in an enhanced accumulation of P-ERK1/2. In contrast, in HASMCs challenged with SU1498, the level of P-ERK1/2 showed a small decrease, or no change, depending on the experiment (Fig.3 and reference (23)). When Elk phosphorylation (*i.e.* the kinase activity) was measured in the lysates in the absence and the presence of 10 μM SU1498, the enzyme activity in

each cell type was sensitive to the inhibitor. Note, however, that the inhibition was much stronger in HUVEC lysates than in HASMC lysates and that the level of phospho-ERK1/2 as well as the kinase activity of ERK in unstimulated cells was considerably higher in HASMCs than in HUVECs. We conclude that the absence of an appreciable effect of SU1498 on HASMCs is not caused by a qualitative distinctiveness of ERKs in these cells but is related to the *phosphorylated* ERKs' abundance.

Human mammary gland epithelial cells - We extended these observations to the four cell lines derived from the human breast epithelium (see **EXPERIMENTAL PROCEDURES**). First, we examined phospho-ERK accumulation in these cells. As shown in Fig. 4, in MCF-10A (a non-tumorigenic cell line), the response to the SU1498 resembled the endothelial cell behavior: phosphorylation of ERKs required the presence of a stimulus (S1P or EGF), and SU149 enhanced the accumulation of P-ERK1/2. In contrast, the moderately invasive MCF-7 cells, while robustly responsive to S1P and EGF, showed no appreciable change in P-ERK1/2 accumulation (Fig. 4 and 5) in the presence of SU1498. The strongly invasive tumor cell lines MDA-MB-231 and MDA-MB-468 possessed highly phosphorylated ERK1/2, the level of which was constant, regardless of the treatment. The elevation of P-ERK1/2 in these cell lines has been attributed to an autocrine loop involving urokinase plasminogen activator and its cognate receptor, uPAR (27-30). Again, we ascribe the insensitivity to SU1498 to the presence of an excess of P-ERK1/2, rather than to some peculiarity in the enzymes themselves.

That the ERK kinases in the tumor cell lines are qualitatively similar to the ERKs from HUVECs and HASMCs was demonstrated when we examined the accumulation of P-ERK1/2 in whole cell lysates from MCF-7 and MDA-MB-231 cells stimulated with S1P and treated with SU1498, PD98059 (MEK inhibitor) and SB203580-iodo (p38 kinase inhibitor). We also determined the kinase activities of ERKs in immunoprecipitates from these lysates. As shown in Fig. 5A, treatment of cells with PD98059 resulted in lowering of the P-ERK1/2 level to that of unstimulated control, regardless of the presence or the absence of SU1498. Thus, as expected, the functioning of MEK is necessary for ERK phosphorylation. Interestingly, SB203580-iodo reduced P-ERK1/2 accumulation in MDA-MB-231 cells but not in MCF-7 cells, a result consistent with the role of p38 MAP kinase in maintaining elevated P-ERK through the action of uPA-uPAR autocrine loop in the highly invasive breast cancer cell lines (27-31). In keeping with these results, ERK activity assays in immunoprecipitates from the above lysates showed that the kinases are fully sensitive to SU1498, and that the active P-ERK1/2 species are essentially absent from the immunoprecipitates obtained after pretreatment of cells with PD98059 (Fig. 5B). Activity was also absent from the immunoprecipitates of MDA-MB-231 cells treated with SB203580-iodo.

SU1498 enhances apoptosis - We noted earlier that extended incubation with SU1498 led to detachment and death of cells (23). As the importance of MAPK signaling pathways in cell proliferation and survival is broadly documented (32-34), and as SU1498 is an effective inhibitor of ERK1/2, we asked whether the cellular death caused by the inhibitor follows, at least in part, an apoptotic pathway. We chose to examine

MCF-7 and MDA-MB-231 human breast epithelial cells. The two cell lines were grown as described in **EXPERIMENTAL PROCEDURES**, and then incubated under starvation conditions (no serum) or in complete media in the absence or the presence of 10 μM SU1498. After 24 hrs, the cells were harvested, labeled with the help of Annexin-V-Fluos staining kit, and analyzed in a flow cytometer. The annexin V-positive cells could be either apoptotic or necrotic, while propidium iodide (PI) labeling (DNA staining) is indicative of loss of cell integrity. Thus, cells that are annexin-positive but PI-negative are considered apoptotic. The results are presented in Fig. 6. Panel *A* shows that in the presence of serum SU1498 had little if any effect on cell viability, but the inhibitor was very cytotoxic when the cells were starved – approximately 75% of cells did not survive the 24 hr treatment with the inhibitor. In panel *B*, the proapoptotic effect of SU1498 was observed in starved cells, but not when serum was present. It is clear that the invasive cell line, MDA-MB-231, is more susceptible to apoptosis (as defined above) than MCF-7 (approximately 4-fold and 2-fold enhancement, respectively). Additional indication that SU1498 may act proapoptotically was obtained in an experiment in which the compound induced the loss of mitochondrial membrane potential, $\Delta\psi_m$ (Fig. 7), an essential step in the apoptotic process (35). This collapse of $\Delta\psi_m$ (observed in every cell line tested) indicates that SU1498 may be able to initiate the intrinsic pathway of programmed cell death.

DISCUSSION

Control of MAP kinase pathways is a critical aspect of cell survival and proliferation. These pathways are involved in positive and negative regulation of gene

expression, cell growth and programmed cell death (33,36). In mammalian cells there exist several distinctly regulated classes of MAP kinases: the extracellular signal-regulated kinases (ERK1/2), the p38 kinases, the Jun amino-terminal kinases, the less well characterized ERK3 and ERK4, and ERK5 and ERK7 (3,37). These MAP kinases are in turn activated by the cognate MAPK kinases, MEK1/2, MKK3/6, MKK4/7, and MEK5 (37). The relationships between these kinases and the manner in which they are properly up- and down-regulated are complex and involve multi-protein interactions, scaffolding structures, and a number of protein phosphatases, all acting in concert to maintain the balance between differentiation and survival pathways (38). The P-ERK phosphatases are especially important in fine-tuning of ERK activities because of their selectivity and specificity (4,8,39-41)

We focused our attention on the effect of the inhibitor, SU1498, on the ERK1/2 activity *in vitro* and on its actions *in vivo* on the cells of human vasculature and on several breast epithelial cell lines. Initially, we noted that HUVECs and human aortic endothelial cells responded to SU1498 by accumulating phosphorylated ERK1/2 (23). At the same time, the compound inhibited the enzyme activity. On the basis of these observations, we postulated that SU1498 prevents dephosphorylation of P-ERK by blocking access to the accumulated phospho-ERK. Alternatively, the compound could act by inhibiting a specific phosphatase. In this report, we determined that the first alternative is largely correct. As shown in Fig. 2, when phospho-ERK2 was incubated with SU1498 and then treated with MKP-1 phosphatase, most of phosphorylated ERK remained

refractory to the phosphatase action. In contrast, pretreatment of the phosphatase with SU1498 had no effect on the enzyme's ability to fully dephosphorylate P-ERK2.

The accumulation of P-ERKs *in vivo* in cells challenged with SU1498 in the presence of a stimulus (S1P or a protein growth factor) appears to be more of an endothelial cell exception than a universal rule. Neither HASMCs nor the breast cancer cell lines we examined showed accumulation of phospho-ERKs under these conditions, perhaps because of high levels of these *phosphorylated* kinases to begin with (Fig. 3 and 4). Similarly, human lung fibroblasts and human dermal fibroblasts showed no accumulation of P-ERKs upon exposure to SU14982 [2]. Thus far, only the non-tumorigenic epithelial cell line MCF-10A resembled HUVECs (Fig. 4). However, in all cell types we tested, the activity of isolated ERKs in lysates or immunoprecipitates was sensitive to the inhibitory action of SU1498. Thus, the observed differences between the cells could be the result of the intrinsic differences in the abundance of *phospho*-ERKs (as suggested above), or they could be caused by distinct regulatory mechanisms involved in maintaining the ERKs in the phosphorylated state. For example, the cells' complement of phospho-ERK-specific phosphatases may vary depending on the particular cell type (42), or the enzymes may be compartmentalized in a unique manner (41). We cannot, of course, exclude the possibility that there are cellular phosphatases that are *not* prevented by SU1498 from accessing the phospho-ERKs; those phosphatases may, indeed, be instrumental in lowering the levels of P-ERK. Given that there are countless species of phosphatases in every cell, we simply do not know at this point which ones might be of relevance.

Many malignant cell lines possess highly elevated levels of P-ERK1/2 (43-46). Our results confirm the involvement of p38 kinase in the maintenance of constitutively high levels of P-ERKs in invasive breast cancer cells, MDA-MB-231 (27,29-31,47) in keeping with the observed role of p38 kinase in stabilizing the uPAR and uPA mRNA (31,47). In MCF-7 cells, ERK phosphorylation is inducible and insensitive to SB203580-iodo; however, in MDA-MB-231, accumulation of P-ERK is much reduced when this inhibitor is present (Fig. 5).

We have obtained preliminary evidence that SU1498 may act proapoptotically on invasive breast cancer cells (Fig. 6). When the cells are incubated in complete medium containing FBS, the inhibitor has no effect on survival. However, under starvation conditions, SU1498 enhances programmed death, as evidenced by the accumulation of Annexin V – positive cells. The effect is considerably stronger in MDA-MB-231 than in the mildly tumorigenic MCF-7 cells. This difference may be the result of the known absence of caspase 3 from of MCF-7 cells (48,49).

Furthermore, SU1498 acted to dissipate mitochondrial membrane potential, $\Delta\psi_m$, in several cell lines (Fig. 7). Presumably, this should lead to the release of cytochrome *c* and activation of caspase 9. The active caspase 9 could in turn cleave and activate the executioner caspases 3, 6 and 7 (11). This result is not inconsistent with the ability of SU1498 to block ERK activity because ERK2 is known to inhibit caspase 9 by phosphorylating it on threonine 125 (50). It is of note that SU1498 is a member of the

tyrphostin family of inhibitors, some of which have previously been found to affect mitochondrial activity (51,52).

We do not know whether SU1498 causes the collapse of $\Delta\psi_m$ directly or through perturbation of the balance between pro- and antiapoptotic proteins of the Bcl family. The inhibition of ERK activity has been shown to down-regulate the expression of Bcl-2, Bcl-X_L and Mcl-1 while leaving intact the expression of apoptosis-promoting proteins Bax and Bak (53). Thus, it is conceivable that SU1498, by inhibiting ERK activity could cause the proapoptotic shift in the equilibrium between these proteins. Similar reasoning may apply to the MAPK-dependent inactivation of the protein Bad (phosphorylated by ERK on Ser112) and protection of Bid against cleavage by caspase 8 (54). Caspase 8 appeared to be a direct target for MAPK-dependent phosphorylation in lymphatic leukemia cells exposed to ionizing radiation (55) and in T cells challenged with the TNF-related apoptosis-inducing ligand (56). In both these instances, the active MAPK pathway protected the cells from disruption of $\Delta\psi_m$.

SU1498 is an intriguing compound with a multifaceted mode of action (22,23). Originally described as a specific VEGF receptor 2 inhibitor (22), this compound possesses activities that are unrelated to the receptor itself. In our hands, SU1498 effects on ERKs are independent of the stimulus used, be it S1P, VEGF, PDGF or EGF (23, this work), and the levels of SU1498 that we have used are at least an order of magnitude lower than those needed to inhibit EGF or PDGF receptor functioning. In this communication, we have elucidated the mechanism by which SU1498 prevents

dephosphorylation of P-ERK1/2 *in vitro*, showed that different cell lines respond differently to treatment with SU1498 *in vivo* (perhaps because of phosphatase activities refractory to SU1498), and presented evidence that the inhibitor may be able to promote apoptosis in human breast cancer cells. The ability of SU1498 to exert these effects, coupled with its anti-VEGF receptor 2 activity (22,57), makes this and related compounds interesting candidates as cytotoxic drugs that may find application in biochemical and pharmacological investigations of signaling pathways (58,59).

REFERENCES

1. Blume-Jensen, P., and Hunter, T. (2001) *Nature* **411**, 355-365.
2. Wess, J. (1997) *Faseb J.* **11**, 346-354.
3. English, J., Pearson, G., Wilsbacher, J., Swantek, J., Karandikar, M., Xu, S., and Cobb, M. H. (1999) *Exp. Cell Res.* **253**, 255-270
4. Sakaue, H., Ogawa, W., Nakamura, T., Mori, T., Nakamura, K., and Kasuga, M. (2004) *J. Biol. Chem.* **279**, 39951-39957
5. Tanoue, T., Noriguchi, T., and Nishida, E. (1999) *J. Biol. Chem.* **274**, 19949-19956
6. Zheng, C. F., and Guan, K. L. (1993) *J. Biol. Chem.* **268**, 16116-15119
7. Muda, M., Boschert, U., Dickinson, R., Martinou, J. C., Martinou, I., Camps, M., Schlegel, W., and Arkinstall, S. (1996) *J. Biol. Chem.* **271**, 4319-4326
8. Munoz, J. J., Tarrega, C., Blanco-Aparicio, C., and Pulido, R. (2003) *Biochem. J.* **372**, 193-201

9. Saxena, M., Williams, S., Brockdorff, J., Gilman, J., and Mustelin, T. (1999) *J. Biol. Chem.* **274**, 11693-11700

10. Denault, J. B., and Salvesen, G. S. (2002) *Chem. Rev.* **102**, 4489-4500

11. Degterev, A., Boyce, M., and Yuan, J. (2003) *Oncogene* **22**, 8543-8567

12. Hengartner, M. O. (2000) *Nature* **407**, 770-776

13. Deas, O., Dumont, C., MacFarlane, M., Rouleau, M., Hebib, C., Harper, F., Hirsch, F., Charpentier, B., Cohen, G. M., and Senik, A. (1998) *J. Immunol.* **161**, 3375-3383

14. Liu, T., Brouha, B., and Grossman, D. (2004) *Oncogene* **23**, 39-48

15. Yu, S. W., Wang, H., Poitras, M. F., Coombs, C., Bowers, W. J., Federoff, H. J., Poirier, G. G., Dawson, T. M., and Dawson, V. L. (2002) *Science* **297**, 259-263

16. Igney, F. H., and Krammer, P. H. (2002) *Nat. Rev. Cancer* **2**, 277-288

17. Sano, H., Uead, Y., Takakura, N., Takemura, G., Doi, T., Kataoka, H., Murayama, T., Xu, Y., Sudo, T., Nishikawa, S., Nishikawa, S., Fujiwara, H., Kita, T., and Yokode, M. (2002) *Am. J. Pathol.* **161**, 135-143

18. Wu-Wong, J. R., Chiou, W. J., and Wang, J. (2000) *J. Pharmacol. Exp. Ther.* **293**, 514-521

19. Germani, A., Di Carlo, A., Mangoni, A., Straino, S., Giacinti, C., Turrini, P., Biglioli, P., and Capogrossi, M. C. (2003) *Am. J. Pathol.* **163**, 1417-1428

20. Takata, R., Fukasawa, S., Hara, T., Nakajima, H., Yamashina, A., Yanase, N., and Mizuguchi, J. (2004) *Cardiovasc. Pathol.* **13**, 41-48

21. Wiesenauer, C. A., Yip-Schneider, M. T., Wang, Y., and Schmidt, C. M. (2004) *J. Am. Coll. Surg.* **198**, 410-421

22. Strawn, L. M., McMahon, G., App, H., Schreck, R., Kuchler, W. R., Longhi, M. P., Hui, T. H., Tang, C., Levitzki, A., Gazit, A., Chen, I., Keri, G., Orfi, L., Risau, W., Flamme, I., Ullrich, A., Hirth, K. P., and Shawver, L. K. (1996) *Cancer Res.* **56**, 3540-3545

23. Boguslawski, G., McGlynn, P. W., Harvey, K. A., and Kovala, A. T. (2004) *J. Biol. Chem.* **279**, 5716-5724

24. Tong, L., Pav, S., White, D. M., Rogers, S., Crane, K. M., Cywin, C. L., Brown, M. L., and Pargellis, C. A. (1997) *Nat. Structural Biol.* **4**, 311-316

25. Brondello, J. M., Brunet, A., Pouyssegur, J., and McKenzie, F. R. (1997) *J. Biol. Chem.* **272**, 1368-1376

26. Cook, S. J., Beltman, J., Cadwallader, K. A., McMahon, M., and McCormick, F. (1997) *J. Biol. Chem.* **272**, 13309-13319

27. Huang, S., New, L., Pan, Z., Han, J., and Nemerow, G. R. (2000) *J. Biol. Chem.* **275**, 12266-12272

28. Montero, L., and Nagamine, Y. (1999) *Cancer Res.* **59**, 5286-5293

29. Ma, Z., Webb, D. J., Jo, M., and Gonias, S. L. (2000) *J. Cell Sci.* **114**, 3387-3396

30. Chen, J., Baskerville, C., Han, Q., Pan, Z. K., and Huang, S. (2001) *J. Biol. Chem.* **276**, 47901-47905

31. Tran, H., Maurer, F., and Nagamine, Y. (2003) *Mol. Cell. Biol.* **23**, 7177-7188

32. Dancey, J., and Sausville, E. A. (2003) *Nat. Rev. Drug Discovery* **2**, 296-313

33. Ballif, A. B., and Blenis, J. (2001) *Cell Growth Diff.* **12**, 397-408

34. Li, S. P., Junttila, M. R., Han, J., Kähäri, V. M., and Westermarck, J. (2003) *Cancer Res.* **63**, 3473-3477

35. Kroemer, G. (2003) *Biochem. Biophys. Res. Commun.* **304**, 433-435

36. Cross, T. G., Scheel-Toellner, D., Henriquez, N. V., Deacon, E., Salmon, M., and Lord, J. M. (2000) *Exp. Cell Res.* **256**, 34-41

37. Chang, L., and Karin, M. (2001) *Nature* **410**, 37-40

38. Kolch, W. (2000) *Biochem. J.* **351**, 289-305

39. Kim, H. S., Song, M. C., Kwak, I. H., Park, T. J., and Lim, I. K. (2003) *J. Biol. Chem.* **278**, 37497-37510

40. Zhou, B., Wang, Z. X., Zhao, Y., Brautigan, D. L., and Zhang, Z. Y. (2002) *J. Biol. Chem.* **277**, 31818-31825

41. Reffas, S., and Schlegel, W. (2000) *Biochem. J.* **352 Pt 3**, 701-708

42. Wang, H. Y., Cheng, Z., and Malbon, C. C. (2003) *Cancer Lett.* **191**, 229-237

43. Jang, B. C., Sanchez, T., Schaefers, H. J., Trifan, O. C., Liu, C. H., Creminon, C., Huang, C. K., and Hla, T. (2000) *J. Biol. Chem.* **275**, 39507-39515

44. Hoshino, R., Chatani, Y., Yamori, T., Tsuruo, T., Oka, H., Yoshida, O., Shimada, Y., Ari-i, S., Wada, H., Fujimoto, J., and Kohno, M. (1999) *Oncogene* **18**, 813-822

45. Ahmed, N., Oliva, K., Wang, Y., Quinn, M., and Rice, G. (2003) *Br. J. Cancer.* **89**, 374-384

46. Xing, C., and Imagawa, W. (1999) *Carcinogenesis* **20**, 1201-1208

47. Han, Q., Leng, J., Bian, D., Mahanivong, C., Carpenter, K. A., Pan, Z. K., Han, J., and Huang, S. (2002) *J. Biol. Chem.* **277**, 48379-48385

48. Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Krammer, P. H., and Peter, M. E. (1998) *Embo J.* **17**, 1675-1687

49. Janicke, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. (1998) *J. Biol. Chem.* **273**, 9357-9360

50. Allan, L. A., Morrice, N., Brady, S., Magee, G., Pathak, S., and Clarke, P. R. (2003) *Nat. Cell Biol.* **5**, 647-654

51. Sagara, Y., Ishige, K., Tsai, C., and Maher, P. (2002) *J. Biol. Chem.* **277**, 36204-36215

52. Soltoff, S. P. (2004) *J. Biol. Chem.* **279**, 10910-10918

53. Boucher, M. J., Morisset, J., Vachon, P. H., Reed, J. C., Laine, J., and Rivard, N. (2000) *J. Cell. Biochem.* **79**, 355-369

54. Holmstrom, T. H., Schmitz, I., Soderstrom, T. S., Poukkula, M., Johnson, V. L., Chow, S. C., Krammer, P. H., and Eriksson, J. E. (2000) *Embo J.* **19**, 5418-5428

55. Shonai, T., Adachi, M., Sakata, K., Takekawa, M., Endo, T., Imai, K., and Hareyama, M. (2002) *Cell Death Differ.* **9**, 963-971

56. Soderstrom, T. S., Poukkula, M., Holmstrom, T. H., Heiskanen, K. M., and Eriksson, J. E. (2002) *J. Immunol.* **169**, 2851-2860

57. Wang, D., Huang, H.-J., Kazlauskas, A., and Cavenee, W. K. (1999) *Cancer Res.* **59**, 1464-1472

58. Nicholson, D. W. (2000) *Nature* **407**, 810-816

59. Dent, P., and Grant, S. (2001) *Clin. Cancer Res.* **7**, 775-783

FOOTNOTES

[1]**Abbreviations used:** *HUVEC*, human umbilical vein endothelial cells; *HASMC*, human aortic smooth muscle cells; *FBS*, fetal bovine serum; *ERK1/2*, extracellular signal-

regulated kinase 1/2 (mitogen-activated protein kinase, MAPK); *p38α/SAPK2*, stress-activated protein kinase; *MEK,* MAPK kinase; *MKP-1*, MAP kinase phosphatase 1 (P-ERK-specific); *S1P*, sphingosine 1-phosphate; *EGF,* epidermal growth factor; *PDGF,* platelet-derived growth factor; *VEGF*, vascular endothelial growth factor; *uPA*, urokinase plasminogen activator; *uPAR*, urokinase plasminogen activator receptor; *SU1498*, VEGF receptor 2 and ERK1/2 inhibitor; PD98059, MEK inhibitor; *SB203580-iodo,* p38 protein kinase inhibitor; *PI*, propidium iodide; $\Delta\psi_m$, mitochondrial membrane potential; *SDS-PAGE*, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; *PBS*, phosphate-buffered saline; *TBST*, Tris-buffered saline containing 0.1% Tween-20.

[2] G. Boguslawski, unpublished observations.

FIGURE LEGENDS

Figure 1. Selectivity of SU1498 action. Purified active ERK2 (2 ng) or p38 kinase (10 ng) were incubated in the kinase reaction buffer without ATP, with or without the indicated amounts of SU1498 or SB203580-iodo. After 10 min incubation at 25° C, the Elk-1 substrate (0.8 μg) and ATP (50 μM) were added and the reactions continued for 30 min at 30° C. Phosphorylated Elk-1 was detected by Western blotting.

Figure 2. SU1498 protects P-ERK2 from dephosphorylation by MKP-1 phosphatase. In *A*, the selectivity of MKP-1 was tested. The indicated amounts of purified MKP-1 were added to the ERK assay mixtures containing P-ERK (2 ng per assay) and the Elk-ATP-kinase buffer in the total volume of 50 μl, either at time 0 or after 30 min of incubation at 30° C. The reactions were stopped with 4X sample buffer

and 10 μl samples were analyzed for the presence of P-Elk by Western blotting. As expected, MKP-1 did not dephosphorylate P-Elk. In *B*, the MKP-1 phosphatase activity was measured as the ability to dephosphorylate P-ERK2. The assays were performed as described under **EXPERIMENTAL PROCEDURES** with 10 units of phosphatase and 5 ng of phosphorylated ERK2 per assay point. At the indicated times, reactions were stopped and samples (6 μl) analyzed for the presence of the residual P-ERK2. Note that 2.5 times more phospho-ERK2 was used in this part of the experiment than in panel *A* or in Fig. 1, which explains the incompletely protective effect of SU1498. Under the same conditions, increasing the concentration of SU1498 to 40 μM prevented the phosphatase from dephosphorylating P-ERK2 in the course of a 30 min incubation (panel *C*).

Figure 3. ERKs from human aortic smooth muscle cells are sensitive to SU1498. T150 flasks were seeded with 2 x 10^6 cells (HUVECs or HASMCs) and incubated overnight. After an overnight starvation, cells were washed once with PBS and overlaid with 4 ml of DMEM. Control flasks received no additions (NT), while cells in other flasks were challenged with 1 μM S1P and 10 μM SU1498 (indicated as **Cell treatments** at the top and the bottom of the Figure.) SU1498 was added to cells 15 min before S1P. Following a 10 min incubation with S1P, cells were lysed and samples of lysates (approximately 20 μg of protein) assayed for kinase activity. The samples were combined with the Elk-ATP-buffer mixture in the total volume of 40 μl and incubated for 30 min at 30° C. Parallel aliquots of the same lysates received 10 μM SU1498 at 10 min prior to the start of the reaction (indicated by a + sign, as appropriate). Note that the P-

ERK level and kinase activity are much higher in HASMCs than in HUVECs, and that there is a significant amount of basal activity even in unstimulated HASMCs.

Figure 4. Differences in phospho-ERK1/2 responsiveness to SU1498 in human mammary epithelial cells. The four cell lines were grown for 24 hrs in 6-well plates (3 x 10^5 cells per well) and starved overnight. The cells were rinsed with PBS and overlaid with 0.4 ml of DMEM. Where indicated, 10 μM SU1498 was added 15 min before the cells were stimulated with 1 μM S1P or EGF (1 ng/ml). The cells were lysed with 100 μl of lysis buffer, the proteins denatured with 4X sample buffer, and 10 μl aliquots were analyzed by SDS-PAGE. Phospho-ERK1/2 and total ERK proteins were detected with appropriate antibodies. Note the excess of ERK1 over ERK2 in MCF-7 cells; the significance of this remains unknown (also see Fig. 5). The asterisks (*) point to differences between the individual cell lines in response to SU1498.

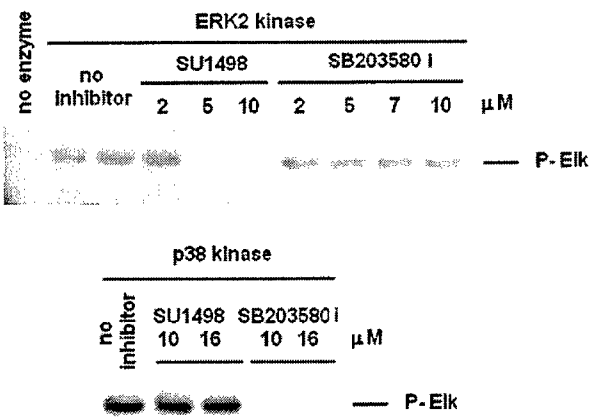
Figure 5. Phospho-ERKs in breast cancer cells are inhibited by SU1498. Accumulation of P-ERK (panel *A*) in tumorigenic cell lines MCF-7 and MDA-MB-231 was examined in whole cell lysates, and the kinase activity (panel *B*) was measured in immunoprecipitates from duplicate aliquots (300 μg of protein) of each lysate, as described in **EXPERIMENTAL PROCEDURES**. Where indicated, 20 μM PD98059 and SB203580-iodo were added to cells 15 min before 10 μM SU1498. Cells were stimulated with 1 μM S1P for 10 min. Following the lysis and immunoprecipitation, the kinase activity in parallel samples was measured (+/- 10 μM SU1498). The phosphorylated Elk1

protein was detected by Western Blotting. Note that in every instance SU1498 is inhibitory, even where the total level of activity is very low.

Figure 6. SU1498 acts proapoptotically on invasive breast cancer cells, MCF7 and MDA-MB-231. To determine the effect of SU1498 on survival, cells were grown in 6-well plates in the presence or the absence of 10 % FBS, with or without 10 μM SU1498 (panel *A*). The wells were washed with PBS, and the attached cells were harvested by trypsinization and counted in a hemocytometer. Separately (panel *B*), cells were grown in T150 flasks under similar conditions, except 2% FBS was present, where indicated. These cells were then analyzed for Annexin V (panel *B*) staining as described in **EXPERIMENTAL PROCEDURES**. In each case, 10,000 events were recorded. The cells showing Annexin V-positive response, but not stained with PI, are considered apoptotic.

Figure 7. SU1498 causes collapse of mitochondrial membrane potential, $\Delta\psi_m$. Cells grown on chamber slides were starved for 24 hrs in the absence or the presence of 10 μM SU1498, as indicated. Following the treatment, the cells were stained with the fluorogenic dye JC-1 and examined for green and red fluorescence (530 nm and 590 nm, respectively). The cells that stain red retain the membrane potential; very few such cells remain in SU1498-treated preparations.

Fig. 1


ERK2 kinase
no enzyme
no inhibitor
SU1498
SB203580 I
2 5 10 2 5 7 10 µM
P-Elk
p38 kinase
no inhibitor
SU1498 SB203580 I
10 16 10 16 µM
P-Elk

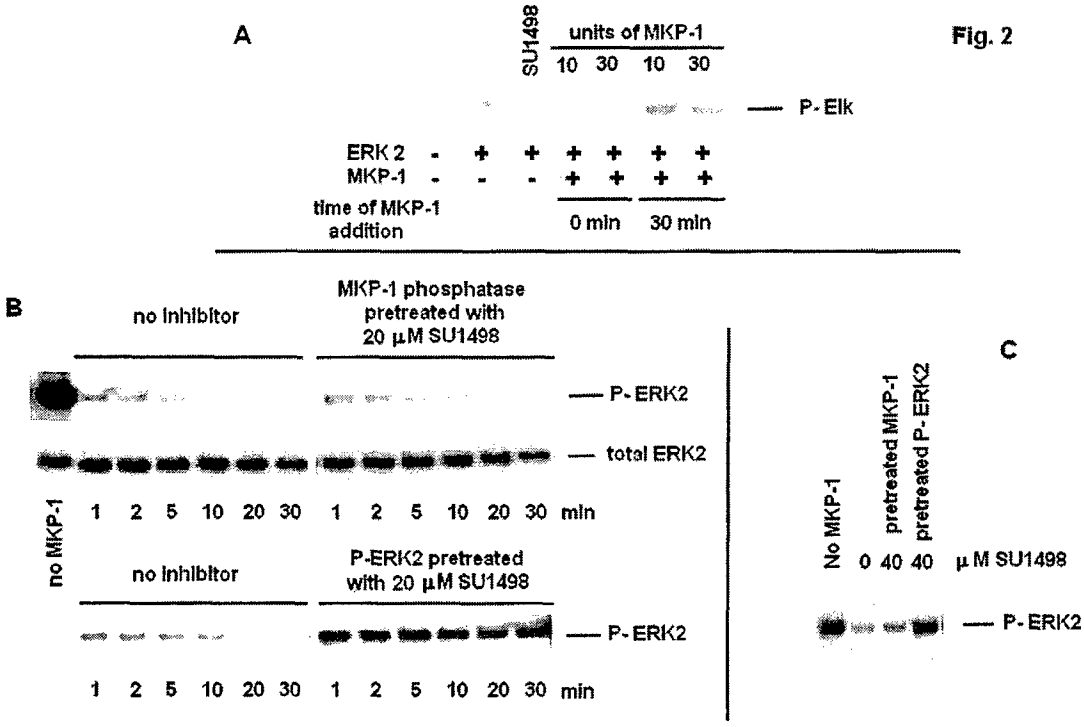

A
SU1498
units of MKP-1
10 30 10 30
P-Elk
ERK 2 - + + + + + +
MKP-1 - - - + + + +
time of MKP-1 addition
0 min 30 min
B
no inhibitor
MKP-1 phosphatase pretreated with 20 µM SU1498
P-ERK2
total ERK2
no MKP-1
1 2 5 10 20 30 1 2 5 10 20 30 min
no inhibitor
P-ERK2 pretreated with 20 µM SU1498
P-ERK2
1 2 5 10 20 30 1 2 5 10 20 30 min
C
No MKP-1
pretreated MKP-1
pretreated P-ERK2
0 40 40 µM SU1498
P-ERK2

Fig. 2

Fig. 3

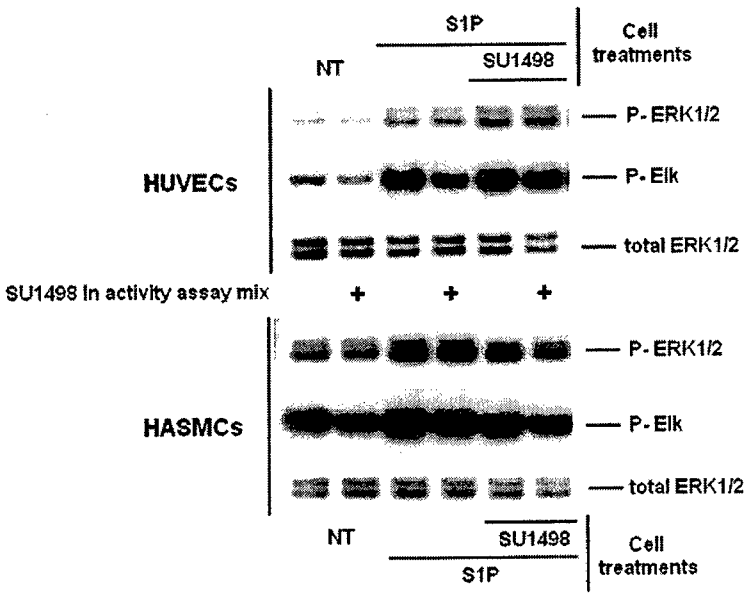

S1P
NT
SU1498
Cell treatments
HUVECs
P-ERK1/2
P-Elk
total ERK1/2
SU1498 in activity assay mix
+
+
+
HASMCs
P-ERK1/2
P-Elk
total ERK1/2
NT
SU1498
S1P
Cell treatments

Fig. 4

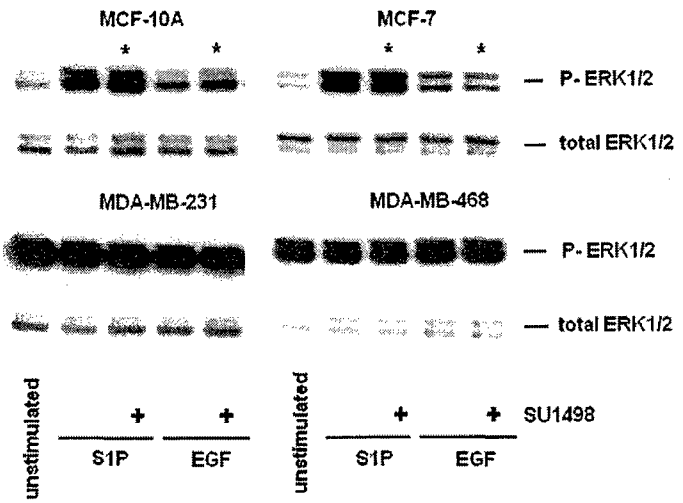
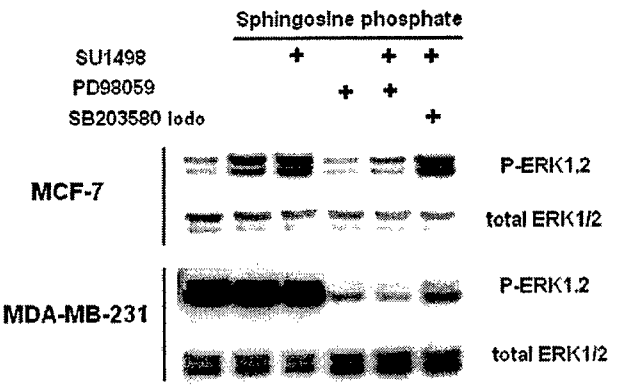
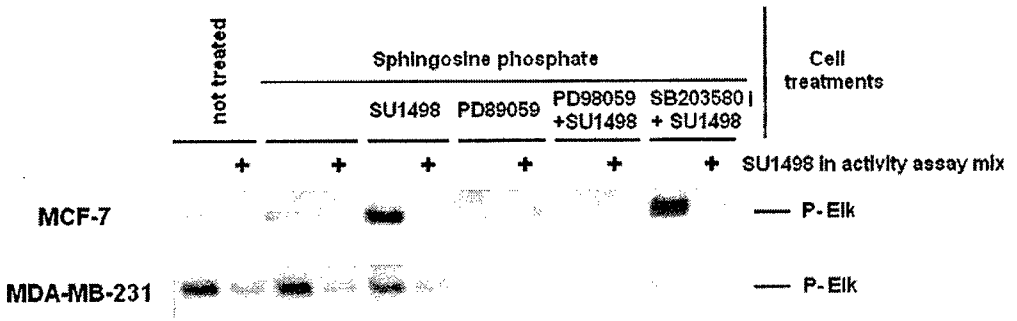

MCF-10A
MCF-7
P-ERK1/2
total ERK1/2
MDA-MB-231
MDA-MB-468
P-ERK1/2
total ERK1/2
unstimulated
S1P
EGF
unstimulated
S1P
EGF
SU1498

Fig. 5

A


Sphingosine phosphate
SU1498
PD98059
SB203580 Iodo
MCF-7
P-ERK1,2
total ERK1/2
MDA-MB-231
P-ERK1,2
total ERK1/2

B


not treated
Sphingosine phosphate
SU1498
PD89059
PD98059 +SU1498
SB203580 + SU1498
Cell treatments
SU1498 in activity assay mix
MCF-7
P-Elk
MDA-MB-231
P-Elk

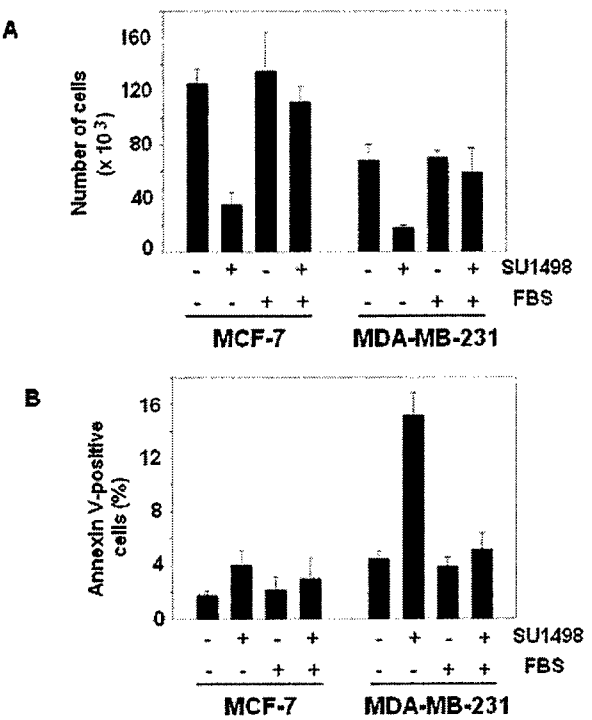
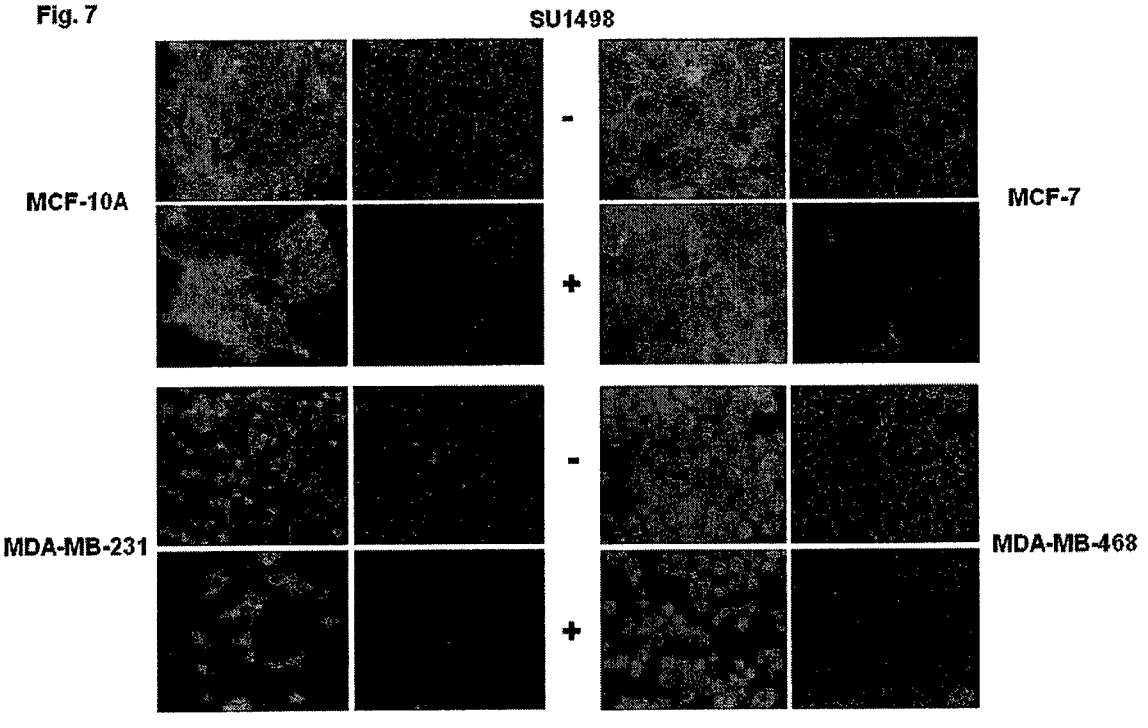

A
160
120
80
40
0
Number of cells (x 10 3)
- + - + - + - + SU1498
- - + + - - + + FBS
MCF-7
MDA-MB-231
B
16
12
8
4
0
Annexin V-positive cells (%)
- + - + - + - + SU1498
- - + + - - + + FBS
MCF-7
MDA-MB-231

Fig. 6

Fig. 7



March 7, 2005
Volume 19, Number 5

THE FASEB JOURNAL

A MULTIDISCIPLINARY RESOURCE FOR THE LIFE SCIENCES

Experimental Biology 2005®

April 2 – 6, 2005

and

XXXV International Congress of Physiological Sciences

March 31 – April 5, 2005

San Diego, California

ABSTRACTS

PART II

Abstracts 568.1 – 1024.3

Official Publication of the Federation of American Societies for Experimental Biology

WOMEN SCIENTISTS' NETWORKING AND MENTORING SESSION/RECEPTION (953.1)

953.1

Women Scientists' Mentoring/Networking Session

Adele J Wolfson[1], Marilee Benore-Parsons[2]. [1]Chemistry Department, Wellesley College, 106 Central Street, Wellesley, MA, 02481, [2]Department of Natural Sciences, Univ Michigan Dearborn, 4901 Evergreen Road, Dearborn, MI, 48128

This panel and reception have become a regular feature of the annual meeting. The session is intended as a forum for discussion of personal histories and strategies for increasing access for women in biochemistry. This year's session will focus on "Programs that Work," proven successes in promoting women in the sciences. Programs that support women in gaining admission to graduate school, in entering the faculty ranks, and in advancing to leadership positions will be discussed. The reception following the panel will provide opportunities for forming mentoring partnerships.

MOLECULAR TARGETS IN DIET AND CANCER II (965.1-965.8)

965.1

Inhibitory Effect of Prolonged-Butyrate Treatment on Migration and Invasion of HT1080 Tumor Cells

Huawei Zeng, Mary Briske-Anderson GF Hum Nutr Res Ctr, USDA, 2420 2nd Ave No, PO Box 9034, Grand Forks, North Dakota, 58202-9034

Butyrate, a normal constituent of the colonic luminal contents, has been hypothesized that butyrate may inhibit the invasion of tumor cells. The present study was to investigate the effects of butyrate on the growth, migration, and invasion characteristics of tumor HT1080 cells. HT1080 cells cultured in the presence of 0.5 and 1 mmol/L butyrate for 14 d exhibited an increase in the G1 and G2 fractions with a concomitant drop in the S-phase, thus showing slower cell growth. Interestingly, 0.5 and 1 mmol/L butyrate inhibited the migration and invasion rate of the tumor cells when compared with the untreated cells. The protein and mRNA levels of the tissue inhibitors of metalloproteinase-1 (TIMP-1) and TIMP-2 were significantly increased in HT1080 cells cultured with 0.5 and 1 mmol/L butyrate. Enzymatic activities and the mRNA level of the latent forms of matrix metalloproteinase (MMP), pro-MMP-2 and pro-MMP-9, were also increased in HT1080 cells cultured with 0.5 and 1 mmol/L butyrate. In contrast, the active MMP-2 was detectable by zymographic analysis in control but not butyrate conditioned media. Collectively, these results demonstrate that prolonged and low-dose butyrate treatment increases both pro-metastasis MMP-2, -9 and anti-metastasis TIMP-1, -2 expression, and the net effect of these increases is the inhibition of pro-MMP-2 activation and of tumor cell migration/invasion potential.

965.2

Omega-3 fatty acids attenuate breast cancer growth through activation of a sphingomyelinase-madiated pathway

Rafat Siddiqui[1], Min Wu[1], Kevin Harvey[1], William Stillwell[2], Gary Zaloga[1] [1], Methodist Research Institute, 1801 N. Capitol Ave, Indianapolis, IN, 46206, [2], Indiana University-Purdue University, 723 W. Michigan Street, Indianapolis, IN, 46202

The effect of fish oils and their active omega-3 fatty acid constituents, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), were investigated on breast cancer growth. *In Vivo Experiments*: Mice were fed diets that were either omega-3-rich (fish oil) or omega-6-rich (corn oil). Three weeks post implantation, the tumor volume and weight decreased significantly ($P<0.05$) for mice fed the omega-3 diets compared to those fed the omega-6 diets. Fish oil also caused increased neutral sphingomyelinase (N-SMYase) activity by 40% ($P<0.05$) in the tumors. *In Vitro Experiments*: DHA and EPA inhibited growth of MDA-MB-231 cells in culture in a dose dependent manner. A concentration as low as 25 μM, DHA and EPA inhibited the growth by up to 20-25% ($P<0.05$). N-SMYase activity was also increased by 70-75% ($P<0.05$) in these cells. In addition, increased ceramide formation was observed in MDA-231-MB cultured cells upon DHA or EPA treatment. Upon fractionation of tumor cell membranes, DHA was shown to enhance N-SMYase activity in the detergent-soluble (non-raft) fractions. DHA and EPA were both observed to enhance membrane bleb formation and also induce the expression of p53 and p21. Blebs were shown to exhibit outer leaflet phosphatidylserine as assayed by annexin V staining. DHA- or EPA-induced bleb formation and apoptosis was inhibited by 40% ($P<0.05$) in the presence of the N-SMYase inhibitor (GW4869). In conclusion, our results suggest that inhibition of breast cancer growth in nude mice by fish oil or inhibition of breast cancer cell growth in culture by treatment with DHA and EPA appear to be mediated by generation of ceramide through enhanced N-SMYase activity.

965.3

The Green Tea Compound EGCG Regulates Wnt Signaling through the HBP1 Transcriptional Repressor

jiyoung kim[1,2], K Eric Paulson[2,3], Amy S Yee[1]. [1]Biochemistry, Tufts Univ., School of Medicine, 136 Harrison Ave., Boston, MA, 02111, [2]Cell and Molecular Nutrition, Tufts Univ., 150 Harrison Ave., Boston, MA, 02111, [3]Radiation Oncology, Tufts-NEMC, 750 Washington St., Boston, MA, 02111

Dysregulation of Wnt signaling has been observed in numerous cancers. In breast cancer, excessive beta-catenin levels are associated with poor prognosis. Cyclin D1, Cox-2, c-MYC and other cancer genes are increased with de-regulated Wnt signaling. Our previous work has identified the HBP1, as a suppressor of Wnt signaling, a repressor of these genes, and an inhibitor of G1 progression. Ongoing work has linked HBP1 to suppression of invasive breast cancer. We reasoned suppressing Wnt signaling might be important for tumor suppression and for cancer prevention. Using the suppression of Wnt signaling as criteria for a screen of phytonutrients, the green tea compound EGCG ((-)-epigallocatechin-3-gallate) was the best candidate. EGCG has been linked to a reduced risk of cancer in animal models and in human breast cancer.

In breast cancer cells, Wnt signaling was inhibited by EGCG in a dose-dependent manner. While the levels of beta catenin were unchanged, the levels of HBP1 were increased with EGCG. DNA-based siRNA was used to knockdown the HBP1 protein, and to assess the consequences for both Wnt signaling and for EGCG suppression. As expected, the knockdown of HBP1 conferred increased sensitivity to Wnt signaling, and higher target gene expression. The HBP1 siRNA breast cell lines had reduced sensitivity to EGCG in the suppression of Wnt signaling. In addition, EGCG reduced the proliferation and invasiveness that characterizes invasive breast cancer. Together, these data suggest that EGCG inhibits Wnt signaling by inducing the HBP1, which could be a potential new biomarker in future prevention studies for invasive breast cancer (supported by NIH and DOD).

965.4

Luteolin inhibits cell proliferation by inducing cell cycle arrest in G1 and G2/M phases in HT-29 human colon cancer cells

Do Y. Lim, Jung H Y Park. Life Sciences, Hallym University, Division of Life Sciences, Hallym University, Chunchon, 200-702, Korea, Republic of

Luteolin is a 3¡¯,4¡¯,5,7-tetrahydroxyflavone found in celery, green pepper and perilla leaf. It has been found to exhibit antimutagenic, antitumorigenic, antioxidant and anti-inflammatory properties. To examine the effect of luteolin on HT-29 cell growth, cells were cultured in the absence or presence of various concentrations of luteolin. Luteolin decreased a viable HT-29 cell number in a concentration dependent manner. The decrease in cell growth was due to an increase in apoptosis and a decrease in DNA synthesis. A G1 phase arrest was induced within 2 h after addition of 60 μM luteolin. Luteolin decreased phosphorylated retinoblastoma proteins (Rb) and cyclin D1, and increased hypophosphorylated Rb levels. However, luteolin did not alter cyclin A, cyclin E, cyclin-dependent kinase (CDK) 2 or 4 levels. The activities of CDK2 and CDK4 were decreased by luteolin in a time dependent manner. A dose-dependent decrease in CDK4 activity was observed within 2 h after the addition of luteolin which correlated with the decrease in cyclin D1 levels. Luteolin also arrested cell cycle progression at G2/M phase following 24 h after the luteolin treatment. Luteolin treatment did not alter phospho-cell division cycle (CDC)2, total CDC2 or phospho-cyclin B1 protein levels. However, cyclin B1 and cdc25C were dose-dependently decreased in concert with increased CDC2 activity. We have demonstrated that lutoelin decreases the HT-29 cell growth by increasing apoptosis and arresting cell cycle progression

965 Molecular Targets in Diet and Cancer II

Minisymposium

Tue. 3:00 PM--Convention Center, 6C

Chair: N. Hord

3:00 PM **965.1** Inhibitory effect of prolonged-butyrate treatment on migration and invasion of HT1080 tumor cells. **H. Zeng and M. Briske-Anderson** USDA, Grand Forks.

3:15 PM **965.2** Omega-3 fatty acids attenuate breast cancer growth through activation of a sphingomyelinase-madiated pathway. **R. Siddiqui, M. Wu, K. Harvey, W. Stillwell and G. Zaloga** Methodist Res. Inst. and Indiana Univ.-Purdue Univ. Indianapolis.

3:30 PM **965.3** The green tea compound EGCG regulates Wnt signaling through the HBP1 transcriptional repressor. **J. Kim, K.E. Paulson and A.S. Yee** Tufts Univ. Sch. of Med. and Tufts-New England Med. Ctr.

3:45 PM **965.4** Luteolin inhibits cell proliferation by inducing cell cycle arrest in G1 and G2/M phases in HT-29 human colon cancer cells. **D.Y. Lim and J.H.Y. Park** Hallym Univ., Republic of Korea.

4:00 PM **965.5** Kaempferol induces cell cycle arrest and apoptosis in HT-29 human colon cancer cells. **H.J. Cho, G.T. Kwon and J.H.Y. Park** Hallym Univ., Republic of Korea.

4:15 PM **965.6** Use of proteomics to identify a covalent protein target of isothiocyanate cancer chemopreventives. **D.J. Templeton and J.V. Cross** Univ. of Virginia.

4:30 PM **965.7** Membrane lipid profiles of exercise-induced weight-control mice. **P. Ouyang, H.M. Doan, D. Vasquez, R. Welti, S-S. Yang and W. Wang** Kansas State Univ.

4:45 PM **965.8** Dietary fish oil may decrease colonic cell proliferation by upregulating p27Kip1 level in actively proliferating cells. **M.Y. Hong, N.D. Turner, M.E. Murphy, R.J. Carroll, R.S. Chapkin and J.R. Lupton** Texas A&M Univ.

Program/Abstract # 965.2

Omega-3 fatty acids attenuate breast cancer growth through activation of a sphingomyelinase-madiated pathway

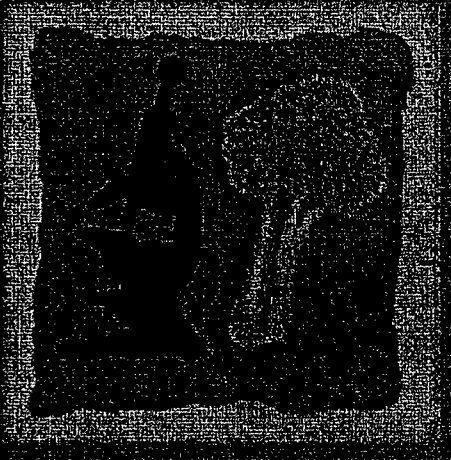
Rafat Siddiqui[1], Min Wu[1], Kevin Harvey[1], William Stillwell[2], Gary Zaloga[1, 1], Methodist Research Institute, 1801 N. Capitol Ave, Indianapolis, IN, 46206, [2], Indiana University-Purdue University, 723 W. Michigan Street, Indianapolis, IN, 46202

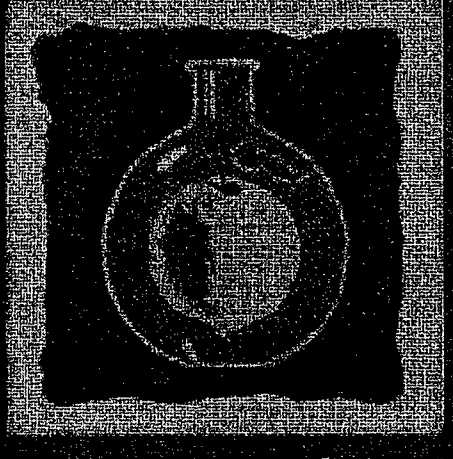
The effect of fish oils and their active omega-3 fatty acid constituents, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), were investigated on breast cancer growth. *In Vivo Experiments*: Mice were fed diets that were either omega-3-rich (fish oil) or omega-6-rich (corn oil). Three weeks post implantation, the tumor volume and weight decreased significantly ($P<0.05$) for mice fed the omega-3 diets compared to those fed the omega-6 diets. Fish oil also caused increased neutral sphingomyelinase (N-SMYase) activity by 40% ($P<0.05$) in the tumors. *In Vitro Experiments*: DHA and EPA inhibited growth of MDA-MB-231 cells in culture in a dose dependent manner. A concentration as low as 25 μM, DHA and EPA inhibited the growth by up to 20-25% ($P<0.05$). N-SMYase activity was also increased by 70-75% ($P<0.05$) in these cells. In addition, increased ceramide formation was observed in MDA-231-MB cultured cells upon DHA or EPA treatment. Upon fractionation of tumor cell membranes, DHA was shown to enhance N-SMYase activity in the detergent-soluble (non-raft) fractions. DHA and EPA were both observed to enhance membrane bleb formation and also induce the expression of p53 and p21. Blebs were shown to exhibit outer leaflet phosphatidylserine as assayed by annexin V staining. DHA- or EPA-induced bleb formation and apoptosis was inhibited by 40% ($P<0.05$) in the presence of the N-SMYase inhibitor (GW4869). In conclusion, our results suggest that inhibition of breast cancer growth in nude mice by fish oil or inhibition of breast cancer cell growth in culture by treatment with DHA and EPA appear to be mediated by generation of ceramide through enhanced N-SMYase activity.

World Cancer Research Fund International

American Institute for Cancer Research

International Research Conference on FOOD, NUTRITION AND CANCER





July 14 & 15, 2005
Hyatt Regency Washington on Capitol Hill
Washington, DC

Neutral sphingomyelinase mediates inhibitory effects of omega-3 polyunsaturation on breast cancer development. Rafat A. Siddiqui[1,2,3] , Min Wu[1], Nargiz Ruzmetov[1], Kevin A. Harvey[1], , Zachary R.Welch[1], Laura Sech[1], Kim Jackson[2], Gary P. Zaloga[1,3], and William Stillwell[2], [1]Cellular Biochemistry Laboratory, Methodist Research Institute, Clarian Health Partners, Indianapolis; [2]Department of Biology, Indiana University-Purdue University, Indianapolis; and [3]Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana.

ABSTRACT

The effect of fish oils and their active omega-3 fatty acid constituents, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), were investigated on breast cancer growth. *In Vitro* Experiments: DHA and EPA inhibited the growth of cultured MDA-MB-231 cells in a dose-dependent manner ($P<0.05$). Neutral sphingomyelinase (N-SMYase) activity was also increased 30-40% ($P<0.05$) in the DHA or EPA treated cells where an increase in ceramide formation was observed. DHA and EPA both enhanced membrane bleb formation and also induced the expression of p21. Both bleb formation and p21 expression were inhibited by the N-SMYase inhibitor GW4869, which also inhibited apoptosis by ~40% ($P<0.05$). *In Vivo* Experiments. Mice were fed diets that were rich in either omega-3 (fish oil) or omega-6 (corn oil) fatty acids. Three weeks after implantation of MDA-MB-231 breast cancer cells, tumor volume and weight were significantly lower ($P<0.05$) for mice fed the omega-3 diet compared to those fed the omega-6 diet. Dietary fish oil also caused a 40% ($P<0.05$) increase in N-SMYase activity in the tumors. The tumor tissues from fish oil–fed animals expressed elevated p21 mRNA, whereas tumor tissues from corn oil–fed did not. DHA and EPA also caused a translocation of N-SMYase activity from plasma membranes to intracellular sites. The results suggest that inhibition of breast cancer cell growth in culture by treatment with DHA or EPA and inhibition of breast cancer growth in nude mice by dietary fish oil is mediated by N-SMYase translocation from membranes to intracellular sites with subsequent enhancement in N-SMYase activity.

Clarian Health
Methodist·IU·Riley

METHODIST RESEARCH INSTITUTE

2004 Summer Student Research Program
Lecture Series

Most lectures will begin at 7:30 a.m. and end at approximately 8:30 a.m. Lectures will meet in the Methodist Research Institute Conference Room, Wile Hall 120, unless otherwise indicated. Please be advised that this schedule is subject to change.

7:30 a.m. MRI Conf Rm Friday, June 4, 2004	Gary P. Zaloga, MD, Medical Director, MRI, "Hypothesis Development"
7:30 a.m. MRI Conf Rm Friday, June 11, 2004	Gary P. Zaloga, MD, Medical Director, MRI, "Research Methdology"
7:30 a.m. Wile Conf Rm Friday, June 18, 2004	Colin Terry, MS, "Introduction to Biostatistics"
8:00 a.m. DG476 South Friday, June 25, 2004	Patty Thurgood, "Using EndNote to Create References" **[This class will be a 2-hour lecture (8 a.m. to 10:00 a.m.)]**
12 noon Noyes Conf Rm Tuesday, July 6, 2004	Wade Clapp, MD, Director, Indiana University School of Medicine MD/PhD Program
7:30 a.m. Wile Conf Rm Friday, July 16, 2004	Steve Miller, PhD, "Writing a Research Paper"
8:00 a.m. MRI Conf Rm Tuesday, July 20, 2004	Group Photo Shoot (Breakfast after in MRI Conference Rm)
7:30 a.m. MRI Conf Rm Friday, July 23, 2004	Karen Spear, PhD, "Ethical Conduct in Research"
7:30 a.m. MRI Conf Rm Friday, July 30, 2004	Karen Spear, PhD, "Preparing an Effective Oral Presentation"